Uterotrophic Actions of Estradiol and Tamoxifen Are Associated with Inhibition of Uterine Insulin-like Growth Factor Binding Protein 3 Gene Expression

Hung Huynh and Michael Pollak
Lady Davis Research Institute of the Jewish General Hospital, and McGill University, Montreal, Quebec H3T 1E2, Canada

Abstract

We have recently shown that uterine insulin-like growth factor I (IGF-I) gene expression is up-regulated by tamoxifen, a uterotropic partial antagonist to the estrogen receptor, but down-regulated by the complete estrogen receptor antagonist ICI 182780, which causes uterine involution. This result is consistent with prior reports indicating that the uterotrophic effects of estradiol are mediated at least in part by estradiol-stimulated uterine IGF-I gene expression. We demonstrate here that the uterotrophic agents estradiol and tamoxifen each suppress expression of the IGF binding protein 3 (IGFBP-3) gene in uterus to less than one-third of control values, while oophorectomy or administration of the complete estrogen receptor antagonist ICI 182780, both of which result in uterine involution, are associated with a greater than 3-fold stimulation of uterine IGFBP-3 gene expression. The data reveal a negative correlation between uterine weight and uterine IGFBP-3 gene expression as well as reciprocal regulation by estradiol of expression in uterus of the genes encoding IGF-I and IGFBP-3. In vitro, IGFBP-3 protein accumulation in media conditioned by primary uterine cultures was decreased by estradiol treatment and increased by ICI 182780 treatment. Together, these observations provide a novel mechanism by which estradiol and antiestrogens modulate uterine IGF-I physiology that is consistent with the view that the mitogenic activity of IGF-I is reduced in the presence of IGFBP-3. The uterotrophic toxicity of chronic estradiol or tamoxifen treatment may be causally related to both the inhibition of uterine IGFBP-3 expression and the stimulation of uterine IGF-I expression by these compounds.

Introduction

IGF-I is a potent mitogen for many normal and neoplastic cell types, including those comprising uterus. Circulating IGF-I is largely hepatic in origin, and growth hormone positively regulates hepatic IGF-I gene expression (1). However, IGF-I bioactivity in various tissues that are targets for IGF-I action is not merely a function of serum levels; there is evidence that local expression of IGF-I and various IGF binding proteins are important in this context and are under complex physiological regulation (2, 3). In the uterus, local expression of the IGF-I gene is stimulated by estrogens, and the positive trophic effect of estradiol on this organ is thought to be mediated at least in part by IGF-I (1). Our recent demonstration (5) that uterine IGF-I gene expression is a molecular marker that correlates with the positive or negative uterotrophic effects of estrogen receptor antagonists and partial agonists is consistent with this model.

IGFBP-3 is the most abundant IGF binding protein in the circulation, where it forms a Mr 150,000 complex with an acid-labile subunit and IGF-I or IGF-II (2). The gene encoding the Mr 33,000–45,000 IGFBP-3 protein is expressed in a variety of tissues, including uterus (reviewed in Ref. 2). IGFBP-3 has been noted in most (but not all) experimental systems to attenuate the mitogenic activity of IGFs (6, 7, and reviewed in Ref. 2). Evidence has also been presented that IGFBP-3 has growth inhibitory activity that is independent of its IGF binding properties (8) and that IGFBP-3 attenuates estradiol-stimulated proliferation of a cell line that is mitogenically responsive to both IGF-I and estradiol (9).

The demonstrated efficacy of tamoxifen in breast cancer treatment has led to widespread therapeutic use of this compound and to ongoing research regarding its potential as a breast cancer preventative agent, but uterotrophic effects and endometrial neoplasia have been associated with tamoxifen therapy (reviewed in Ref. 5). Because insight into the molecular mechanisms underlying the uterine toxicity of tamoxifen may be relevant to the design of improved endocrine therapies for breast cancer, the present studies were undertaken to test the hypothesis that the uterotrophic actions of estrogens and tamoxifen are associated with suppression of uterine IGFBP-3 expression.

Materials and Methods

Animals, Drug Administration, and Sample Collection. Animal experiments were approved by McGill University Animal Care Committee. Fifty-day-old pituitary intact, OVX, and hypox Sprague-Dawley female rats (Charles River) were used. Ablative surgery was carried out at least 2 weeks prior to beginning experiments. We studied intact control, intact tamoxifen-treated, intact ICI 182780-treated, hypox control, hypox tamoxifen-treated, ICI 182780-treated, hypox-growth hormone replaced, hypox estrogen-replaced, OVX control, and OVX-estrogen replaced animals for a total of 10 experimental groups. Each experimental group consisted of four animals. Tamoxifen and ICI 182780, a complete antagonist to the estrogen receptor (10), were administered using a dose and route previously shown to have inhibitory effect on 7,12-dimethylbenz(a)anthracene-induced mammary tumors. 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 s.c. administered 0.2 ml peanut oil. All the animals were sacrificed by CO2 exposure 7 days after the first day of treatment. For the growth hormone replacement experiments, hypox rats were administered 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. To obtain a constant release of 1 µg estradiol/day, groups of OVX or hypox rats were implanted with 0.4-cm silastic tubes (0.04 in. inside diameter; Dow Corning, Midland, MI) containing 17β-estradiol on the back of their neck. Control rats had the same surgical implantation with empty silastic tubes. Based on previously published work (11), the release rate of 17β-estradiol from silastic implants was documented to be 2.4 µg/cm/day. Based on dose-
All animals were sacrificed by carbon dioxide exposure on day 8. The uteri were excised, immediately frozen in liquid nitrogen, weighed, and stored at −75°C for subsequent RNA extraction.

**RNA Extraction and Hybridization.** Total RNA was isolated from uteri using RNAzol premix solution and RNAzol B method (Tel-Test, Friendswood, Texas). 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 IGFBP-3 complementary DNA (12) (kindly provided by Dr. S. Shimasaki, Whittier Institute, San Diego, CA), 10% dextran sulfate, 1% SDS, 500 μg/ml herring sperm DNA, 0.9 mM NaCl, 50 mM Na2HPO4−7H2O, and 5 mM EDTA. The membranes were subjected to three washes at 42°C 15 min each in solution A (2X SSC-0.1% SDS), solution B (0.5X SSC-0.1% SDS), solution C (0.1X SSC-0.1% SDS), respectively. 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 gels, we compared total amounts of RNA present in different lanes by rehybridizing the blots with labeled rat 18S ribosomal RNA kindly provided by Dr. C. Karatzas (McGill University, Montreal, Canada). Quantitative analysis of gene expression was accomplished by scanning autoradiographs densitometrically. For each lane, only the density of the band corresponding to the 2.6-kilobase IGFBP-3 transcript was used, and this figure was adjusted to show that the mean density of the 2.6-kilobase band representing the IGFBP-3 transcript was used, and this figure was adjusted to show that the mean density of the band corresponding to the 2.6-kilobase IGFBP-3 transcript was used, and this figure was adjusted to show.
complete estrogen receptor antagonist that causes uterine involution (10), stimulates IGFBP-3 gene expression more than 3-fold over control. The low molecular weight IGFBP-3 mRNAs seen in Fig. 1 were not due to mRNA degradation or nonspecific binding and were consistently observed in this tissue.

Because IGFBP-3 serum levels are effected by hypophysectomy (17) and estrogens and antiestrogens have important actions on the pituitary including modulation of GH secretion (18, 19), we considered the possibility that the pituitary might be involved in the effects of the antiestrogens on regulation of uterine IGFBP-3 gene expression. Data presented in Fig. 2 demonstrate that hypophysectomized animals exhibit a high level of uterine IGFBP-3 gene expression (consistent with uterine involution and an estrogen-deficient state). Estradiol and tamoxifen each suppress uterine IGFBP-3 gene expression in hypophysectomized rats, and GH replacement has no significant effect on uterine IGFBP-3 gene expression in these animals. ICI 182780 did not further stimulate the already elevated levels of IGFBP-3 expression in uteri of hypophysectomized rats (data not shown). These data suggest that the inhibitory action of estradiol and tamoxifen on uterine IGFBP-3 gene does not involve GH or other pituitary hormones.

To extend our in vivo results, we used a short-term primary uterine tissue culture system. While accurate quantification of proliferation is not possible in this system, we collected serum-free conditioned media to allow quantification of IGF binding proteins by ligand blotting. As shown in Fig. 3, we observed that ICI 182780 increases IGFBP-3 accumulation in a dose- and time-dependent manner with maximum activity seen at 10^-9 m. In contrast, estradiol exposure resulted in decreased IGFBP-3 accumulation compared to control cultures. Thus, the direction of change in IGFBP-3 protein accumulation associated with estradiol or ICI 182780 exposure in vitro is consistent with the direction of change in IGFBP-3 mRNA accumulation observed in vivo. Changes in IGFBP-3 mRNA accumulation in the short-term cultures were in the same direction as changes in IGFBP-3 protein accumulation (data not shown).

Fig. 4 presents relationships between uterine weight, uterine IGF-I gene expression, and uterine IGFBP-3 gene expression in intact animals treated with vehicle, the positive uterotrophic agent tamoxifen, or ICI 182780, which causes uterine involution. The positive correlation between IGF-I gene expression and uterine weight, the negative correlation between IGFBP-3 gene expression and uterine weight, and the negative correlation between IGF-I gene expression and IGFBP-3 gene expression are each statistically significant (P < .001). The IGF-I gene expression data have recently been presented (5).

**Discussion**

The facts that estradiol stimulates uterine growth, that estradiol up-regulates uterine IGF-I gene expression, and that various cell types that comprise the uterus are mitogenically responsive to IGF-I provide a basis for the view that IGF-I is an important mediator of the uterotropic effects of estradiol (reviewed in Ref. 4). Our recent demonstration (5) that administration of a complete antagonist to the estrogen receptor is associated with both uterine involution and a decrease in uterine expression of the IGF-I gene is consistent with this hypothesis. However, we suspected that there might be additional mechanisms besides modulation of IGF-I gene expression by which estradiol stimulates and antiestrogens inhibit uterine growth. More specifically, we speculated that estradiol might suppress expression of a molecule that reduces IGF-I bioactivity and that estrogen deprivation might stimulate its expression. We selected IGFBP-3 as a candidate molecule in this regard because we (9, 13) and others (6, 7) have observed that high levels of IGFBP-3 attenuate the mitogenic effect of IGF-I and/or are negatively correlated with proliferation in many experimental systems.

Our results show that oophorectomy enhances uterine IGFBP-3 gene expression and that this is reversed by estradiol replacement. Consistent with this observation, tamoxifen administration, which is associated with uterine hypertrophy, suppresses uterine IGFBP-3 gene expression, while administration of the complete estrogen receptor antagonist ICI 182780 is associated with uterine involution and enhanced IGFBP-3 gene expression. Measurements of IGFBP-3 accumulation in the conditioned media of primary uterine cultures were consistent with changes in IGFBP-3 gene expression measured in vivo. Taken together, our data demonstrate both a negative correlation between uterine IGFBP-3 gene expression and uterine weight and reciprocal regulation of uterine expression genes encoding IGFBP-3 and IGF-I by estradiol, thereby providing an additional molecular mechanism by which estradiol effects intrauterine IGF-I physiology. The relative importance of down-regulation of IGFBP-3 gene expression and up-regulation of IGF-I gene expression in the mediation of estradiol effects cannot be determined from our data nor from the prior studies (reviewed in Ref. 4) that documented the effect of estradiol on uterine IGF-I gene expression. In the light of our findings, it is possible that the uterotrophic effect of estradiol previously attributed to up-regulation of IGF-I gene expression is related in part to the down-regulation of IGFBP-3 gene expression.
It has previously been observed (reviewed in Ref. 4) that, in uterine tissue cultures, exogenous IGF-I enhances DNA replication only in the presence of estradiol, a result not in keeping with the model that the uterotrophic activity of estradiol is entirely attributable to up-regulation of local IGF-I gene expression. While there are several possible explanations for this observation, in light of the data reported here, it is possible that exogenous IGF-I is without effect in the absence of estrogens, because under this condition, abundant IGFBP-3 is present; when estradiol is provided, IGFBP-3 gene expression is down regulated, and the bioactivity of exogenous (or locally produced) IGF-I would become sufficient to result in mitogenic stimulation.

No estrogen response element has been demonstrated on the promoter region of the IGFBP-3 gene (2). Therefore, the precise mechanism of IGFBP-3 regulation by estradiol remains uncharacterized. There is a similar gap in knowledge regarding the precise mechanism of estradiol effect on uterine IGF-I gene expression; although the effect of estradiol on uterine IGF-I gene expression is clear (reviewed in Ref. 4), no estrogen response element has been demonstrated in the action of estradiol is indirect and involves regulation of an estrogen-responsive gene, which in turn regulates IGF-I and IGFBP-3 gene expression. In any case, our results provide an example of low-level constitutive expression of a gene that can be enhanced by ICI 182780 or estrogen deprivation and suppressed by estradiol. ICI 182780 stimulates IGFBP-3 gene expression and protein accumulation in vitro, even under estrogen-free culture conditions. This implies that ICI 182780 may not merely act in a passive fashion to block estrogen-receptor interactions but rather actively regulates gene transcription in a direction opposite to that of estrogens.

Effects of antiestrogens on IGFBP-3 physiology may not be limited to the uterus. We recently observed that ICI 182780 and to a lesser extent tamoxifen increase accumulation of autocrine-produced IGFBP-3 in conditioned media of MCF7 breast cancer cells (9). As recombinant human IGFBP-3 suppresses proliferation of these cells (13), these data are compatible with the hypothesis that IGFBP-3 mediates certain growth inhibitory actions of antiestrogens by decreasing responsivity of breast cancer cells to insulin-like growth factors. Given the well-described tissue specificity of estrogen receptor agonist/antagonist actions of tamoxifen, it is not surprising that this compound increases IGFBP-3 accumulation and inhibits proliferation of breast cancer cells, despite the fact it has opposite effects on uterine tissue cultures, whereas ICI 182780 increases IGFBP-3 accumulation in the conditioned media of all

---

**Fig. 3.** Effect of estradiol, tamoxifen, and ICI 182780 on IGFBP-3 accumulation in the conditioned media of primary uterine cell cultures. Short-term primary uterine cell cultures were prepared as described in "Materials and Methods." Control media were estrogen and serum free. Cultures were exposed to vehicle alone or various concentrations of ICI 182780, estradiol, or tamoxifen as indicated. IGFBP-3 accumulation was estimated by densitometric scanning of the M, 33,000–45,000 band of conditioned media as described in "Materials and Methods." A, ligand blotting of media conditioned by control (C) and ICI-182780 treated primary uterine cells. Cells were treated at various concentrations of ICI 182780 as indicated below each lane. Quantitative analysis of IGFBP-3 accumulation was carried out by densitometric scanning of the M, 33,000–45,000 band corresponding to IGFBP-3 in three ligand blots obtained from three replicate experiments and revealed that maximal accumulation of IGFBP-3 occurred at the concentration 10⁻⁹ M ICI 182780, 470 ± 18% of the control value. B, ligand blotting of media conditioned by primary uterine cells treated with vehicle (C), ICI 182780 (ICI), or 17β-estradiol (E2). Cells were grown in media containing vehicle only, 10⁻⁹ M ICI 182780, or 10⁻⁹ M 17β-estradiol for 24 h, and ligand blots were performed as described in "Materials and Methods." Quantitative analysis of IGFBP-3 accumulation in three independent replicate experiments was carried out as described above and revealed that at 6 h, there was no significant difference between vehicle and ICI 182780-treated cultures but that at 24 h, vehicle-treated cultures had a 320 ± 12% increase over the 6-h value, whereas cells treated with ICI 182780 had a 696 ± 14% increase over the 6-h value. C, ligand blotting of media conditioned by primary uterine cells treated with vehicle (C), ICI 182780 (ICI), or 17β-estradiol (E2). Cells were grown in media containing vehicle only, 10⁻⁹ M ICI 182780, or 10⁻⁹ M 17β-estradiol for 24 h, and ligand blots were performed as described in "Materials and Methods." Quantitative analysis of IGFBP-3 accumulation in three independent experiments was carried out as described above and revealed that ICI 182780 treatment resulted in IGFBP-3 accumulation to 202 ± 14% of the control value, whereas estradiol treatment resulted in IGFBP-3 accumulation to 77 ± 4% of the control value.
This figure summarizes data concerning the effects of tamoxifen treatment and ICI 182780 treatment on uterine weight, uterine IGF-I expression, and uterine IGFBP-3 expression. Treatments were administered, tissues were processed, and mRNA was extracted as described in "Materials and Methods." Each group (control, tamoxifen treated, and ICI 182780 treated) comprised four animals, and mean values are plotted. Within-group variability regarding weight, IGF-I expression, and IGFBP-3 expression were each less than 15%. Three independent experiments gave similar results.

cell types studied. It is of interest that the antiproliferative activity of retinoids on breast cancer cells is also associated with increased IGFBP-3 accumulation (20). Finally, our results suggest a novel mechanism of action for the antiproliferative actions of luteinizing hormone-releasing hormone analogues seen in many experimental systems (21); these compounds, by producing a functional gonadal steroid, may also up-regulate tissue IGFBP-3 expression.

It is recognized that gonadal steroids play important roles in modulating growth hormone output and IGF-I gene expression (22). The fact that estradiol and antiestrogens have important effects on IGFBP-3 gene expression in the uterus raises the possibility that, in general, gonadal steroids also regulate responsivity of certain tissues to insulin-like growth factors by influencing IGF binding protein physiology.

Acknowledgments

We thank Dr. S. Shimasaki for the rat IGFBP-3 complementary DNA, Dr. Alan Wakeling for ICI 182780 and helpful comments, and all those who provided constructive criticism of the manuscript.

References


