

In Vivo Inhibition of Insulin-like Growth Factor I Gene Expression by Tamoxifen¹

Hung T. Huynh, Evelyn Tetenes, Laurie Wallace, and Michael Pollak²

Departments of Medicine and Oncology, McGill University [M. P.], and the Lady Davis Institute for Medical Research of the Sir Mortimer B. Davis-Jewish General Hospital [M. P., H. T. H., E. T., L. W.], Montréal, Québec, Canada

Abstract

Tamoxifen, a partial antagonist to the estrogen receptor, is widely used in the treatment of breast cancer and is currently being evaluated as a breast cancer preventative agent in large-scale clinical trials. Recent clinical research has demonstrated that tamoxifen administration is associated with a reduction of serum insulin-like growth factor I (IGF-I) concentration. We demonstrate here that tamoxifen, when administered in an *in vivo* experimental system previously used to demonstrate its cytostatic effect on breast cancer cell proliferation, inhibits the expression of the IGF-I gene in common target organs for breast cancer metastasis. Furthermore, while our prior experimental studies have demonstrated an inhibitory effect of tamoxifen on growth hormone output, we show here for the first time that the suppression of IGF-I gene expression associated with tamoxifen administration is in part a consequence of a pituitary-independent action of the drug. Because IGF-I is a potent mitogen for breast cancer cells, this newly described activity of tamoxifen may contribute to its antineoplastic properties, particularly with regard to inhibition of metastasis seen both in animal models and clinically.

Introduction

In the past decade, tamoxifen has become one of the most widely used treatments for breast cancer (1); it is estimated that the drug has been prescribed to more than a million women worldwide. In many patients, it causes regression or stabilization of macroscopic metastasis (1), and it prolongs survival and disease-free survival of significant subsets of breast cancer patients when used as a postsurgical adjuvant treatment by inhibiting the proliferation of micrometastases (2). Large-scale clinical trials designed to determine the worth of tamoxifen as a breast cancer preventative agent are in progress (1).

Evidence that IGF-I³ promotes the proliferation of human breast cancer includes the *in vitro* mitogenic response of breast cancer cells to nanomolar IGF-I concentrations, the presence of type 1 IGF receptors in both cloned breast cancer cell lines and primary human breast cancers, and the inhibition of *in vivo* proliferation of breast cancer by a blocking antibody directed against the type 1 IGF receptor (reviewed in Ref. 3). There is also evidence to support the view that IGF-I stimulates the motility and metastatic behavior of malignant cells (4, 5). Autocrine expression of IGF-I by breast cancer cells is uncommon (6); the IGF-I level in the microenvironment of tumor cells is influenced by local production by neighboring cells (including stromal tissue) and by circulating IGF-I, which is mainly hepatic in origin. Although GH is known to be a potent stimulator of IGF-I expression in hepatocytes (7), factors controlling IGF-I expression in extrahepatic tissues are incompletely described.

At least four reports (reviewed in Ref. 3) have confirmed our placebo-controlled study (8), which documented that tamoxifen administration in humans is associated with a reduction in serum IGF-I concentration. Recent *in vivo* and *in vitro* experimental studies (9, 10) have documented an inhibitory effect of tamoxifen on growth hormone secretion, an action that might explain at least in part this clinical observation. In the present study, we used an *in vivo* model system previously used to describe the antineoplastic activity of tamoxifen (11) to allow us to characterize for the first time in intact animals the effect of this commonly used drug on IGF-I gene expression in liver and lung, two important target tissues for breast cancer metastasis. In addition, we carried out experiments with hypophysectomized and hypophysectomized, growth hormone-replaced animals to determine if the clinically observed reduction of IGF-I levels associated with tamoxifen treatment can be entirely attributed to inhibition of growth hormone output by the drug.

Materials and Methods

Animals, Drug Administration, and Sample Collection. Pituitary-intact and hypophysectomized Sprague-Dawley female rats that were 50 days old at the beginning of the experiments were purchased from Charles River (Montréal, Québec, Canada), after approval of this study by the local animal care committee. Rats were housed on a 12-h light, 12-h dark cycle in a temperature- and humidity-controlled room and were fed with Purina rat chow and tap water *ad libitum*. Tamoxifen (Sigma, St. Louis, MO) was administered using a dose and route previously shown to have an inhibitory effect on 7,12-dimethylbenz-(a)anthracene-induced mammary tumors (11). The rats were given s.c. injections of 5 mg tamoxifen in 0.2 ml peanut oil once daily on 2 consecutive days. Control rats were administered 0.2 ml peanut oil s.c. at the same time points. For the growth hormone replacement experiments, rats were not only treated with tamoxifen or peanut oil as described above but also were administered either i.p. saline or i.p. human recombinant growth hormone (kindly provided by Genentech, South San Francisco, CA) at 100 µg/100 g body weight dissolved in saline daily for 7 days, starting on the same day as the tamoxifen or peanut oil. On day 8, blood samples were collected by cardiac puncture and centrifuged, and the serum was separated and stored at -20°C for the subsequent assay of IGF-I. Also on day 8, organs of interest were excised, sectioned into small pieces, and frozen in liquid nitrogen for subsequent Northern blot analysis.

IGF-I Gene Expression. RNA was extracted and analyzed by Northern blot with a ³²P-labeled probe complementary to rat prepro-IGF-I mRNA (12) that was kindly provided by Dr. L. Murphy. Total RNA was isolated using RNeasy lysis solution and RNeasy spin column (Qiagen, Crawfordsville, IN). Fifty µg of total RNA extracted from liver or 60 µg of total RNA extracted from lung were 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 complementary DNA (12), 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× standard saline citrate; 0.1% SDS), solution B (0.5× standard saline citrate; 0.1% SDS), and solution C (0.1× standard saline citrate; 0.1% SDS), respectively. The final wash was carried out at 60°C in solution C. The blots were air-dried and subjected to autoradiography for 1 to 3 days with intensifying screens at -80°C. To control for equal loading of wells, we compared the total amounts of RNA present in different lanes by rehybridizing the blots with labeled β-actin complementary

DNA (13). Quantitative analysis of gene expression 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). In no case was this correction greater than 10%. The densitometric quantification data given in each of Figs. 1B, 2B, and 3B have been corrected for any minor differences in total RNA present in each lane.

IGF-I Radioimmunoassay. Prior to radioimmunoassay, extraction of IGF binding proteins from the sera was carried out by initial acidification with 0.01 N HCl. Following a brief centrifugation to remove high-molecular-weight proteins, free IGF-I in the clarified sera was eluted by sephadex G-75 column chromatography. Fractions known to contain IGF-I were pooled, and IGF-I levels were measured by double antibody radioimmunoassay as previously described (8).

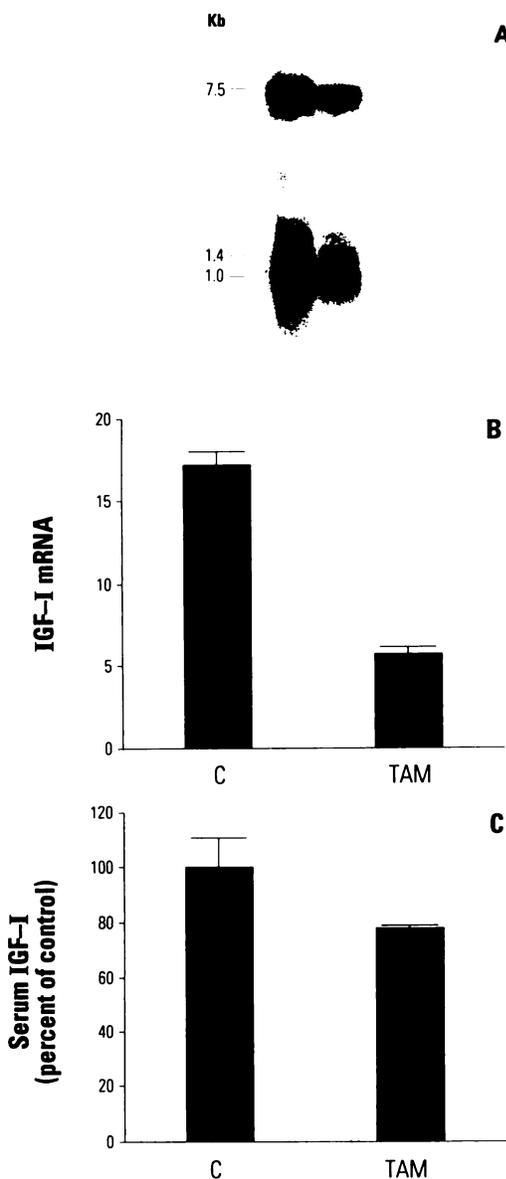


Fig. 1. Effect of tamoxifen on hepatic IGF-I expression in intact rats. **A**, autoradiography of accumulated rat hepatic IGF-I mRNA. *Left lane*, control animal; *right lane*, animal treated with tamoxifen as described in "Materials and Methods." **B**, densitometric quantitation of hepatic IGF-I gene expression in the tamoxifen-treated and control rats. *Columns*, mean of three independent determinations on each of three animals; *bars*, SEM. **C**, serum IGF-I concentration after tamoxifen administration to intact rats. Extraction of IGF-binding proteins from the sera and radioimmunoassay were carried out as described in "Materials and Methods." The mean control serum IGF-I concentration was 1155 ng/ml. *Columns*, mean of three independent experiments on each of three animals; *bars*, SEM.

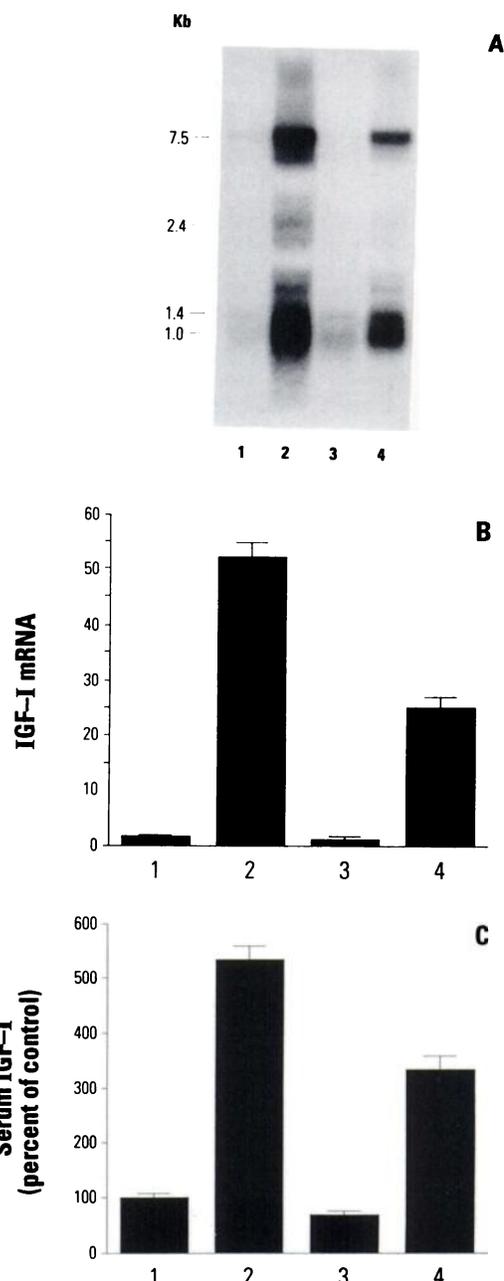


Fig. 2. Effect of tamoxifen on hepatic IGF-I expression in hypox and hypox-GH rats. **A**, autoradiography of accumulated rat hepatic IGF-I mRNA in vehicle-treated, hypox (*Lane 1*); hypox-GH (*Lane 2*); tamoxifen-treated, hypox (*Lane 3*); and hypox-GH (*Lane 4*) rats. Details of animal treatments and Northern blotting are as described in "Materials and Methods." **B**, densitometric quantitation of hepatic IGF-I gene expression in each of the four groups as described above. *Columns*, mean of three independent experiments on each of three separate animals; *bars*, SEM. **C**, serum IGF-I concentration in each of the four groups described above. Extraction of IGF binding proteins from the sera and radioimmunoassay was carried out as described in "Materials and Methods." The mean control serum concentration in hypox controls was 157 ng/ml. *Columns*, mean of three independent experiments on each of three animals; *bars*, SEM.

Results

We first examined the effect of tamoxifen treatment on IGF-I expression in the livers of intact female rats. The data in Fig. 1 (**A** and **B**) demonstrate an approximately 3-fold reduction in IGF-I expression in tamoxifen-treated rats relative to the vehicle-treated controls. The tamoxifen treatment was also associated with an approximately 25% decline in serum IGF-I levels (Fig. 1C), a result in keeping with the degree of decline in serum IGF-I seen in breast cancer patients treated with tamoxifen (8).

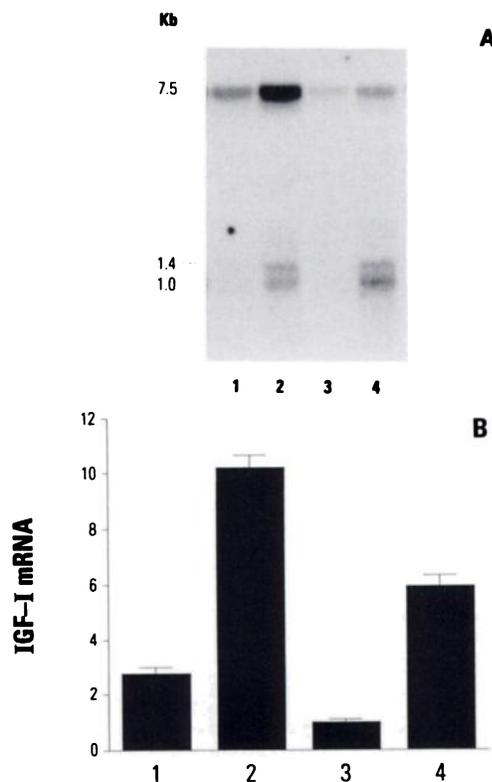


Fig. 3. Effect of tamoxifen on pulmonary IGF-I expression in hypox and hypox-GH rats. A, autoradiography of accumulated rat pulmonary IGF-I mRNA in vehicle-treated, hypox (Lane 1); hypox-GH (Lane 2); tamoxifen-treated, hypox (Lane 3); and hypox-GH (Lane 4) rats. Details of animal treatments and Northern blotting are as described in "Materials and Methods." B, densitometric quantitation of pulmonary IGF-I gene expression of each of the four groups as described above. Columns, mean of three independent experiments on each of three separate animals; bars, SEM.

Because growth hormone (7) and (in rat) prolactin (14) stimulate IGF-I expression, we used hypox and hypox-GH rats in experiments designed to determine if the effect of tamoxifen on IGF-I expression could be attributed entirely to the previously described inhibitory actions of the drug on the hypothalamic-pituitary axis with respect to lactogenic hormone output (9, 10, 15). Northern analysis revealed that hepatic IGF-I mRNA expression in hypox rats was low but detectable and was even further reduced (1.75 ± 0.12 versus 1.24 ± 0.03 density units) in tamoxifen-treated hypox rats (Fig. 2, A and B). This observation raised the possibility of a pituitary-independent suppression of IGF-I gene transcription by tamoxifen. This phenomenon was investigated further with hypox-GH rats. As expected, these animals exhibited high IGF-I mRNA levels compared to saline-treated hypox rats (Fig. 2A, Lane 1 versus Lane 2). The growth hormone-induced elevation in hepatic IGF-I expression was attenuated in tamoxifen-treated hypox-GH rats (Fig. 2A, Lane 4 versus Lane 2). Quantitative analysis of these Northern blots is summarized in Fig. 2B. These data provide further evidence that apart from the previously described inhibitory effect of tamoxifen on lactogenic hormone output (9, 10, 15), the drug has a pituitary-independent inhibitory effect on IGF-I gene expression.

To ascertain whether serum IGF-I levels correlated with changes in IGF-I expression induced by tamoxifen in each of the experimental conditions described above, we determined the concentration of serum IGF-I by radioimmunoassay of samples preprocessed to remove IGF-I binding proteins. Serum IGF-I levels in tamoxifen-treated hypox rats were about 70% of those in control hypox rats (Fig. 2C). There was an approximately 5-fold increase in serum IGF-I following growth hormone replacement but only an approximately 3-fold increase in tamoxifen-treated hypox-GH rats.

To determine if the pituitary-independent inhibitory effect of tamoxifen on IGF-I gene expression was specific to liver, we carried out analogous experiments in which we examined IGF-I expression in the lungs of control and tamoxifen-treated hypox and hypox-GH rats. As expected, baseline IGF-I expression in lung was lower than that in liver. Northern hybridization showed clearly that tamoxifen suppressed pulmonary IGF-I gene transcription in hypox rats (Fig. 3A, Lane 1 versus Lane 3). Growth hormone increased IGF-I mRNA levels in both control and tamoxifen-treated animals, but this effect was obviously attenuated in the tamoxifen-treated hypox-GH rats (Fig. 3A, Lane 2 versus Lane 4). Quantitative analysis (Fig. 3B) indicated that tamoxifen-treated hypox rats had 2.5-fold less pulmonary IGF-I mRNA than vehicle-treated controls. Growth hormone increased pulmonary IGF-I expression in control hypox rats almost 5-fold, but this effect of growth hormone was obviously attenuated in tamoxifen-treated hypox-GH rats. This provides evidence for a pituitary-independent inhibitory effect of tamoxifen on IGF-I expression in lung.

Discussion

The classic model of the mechanism of action of tamoxifen emphasizes direct actions of the drug at the level of estrogen receptor-positive neoplastic cells (1). The data reported here suggest that tamoxifen has, in addition, an effect on gene expression by certain host organs which would be expected to result in a less favorable microenvironment for the proliferation of IGF-I-responsive metastatic cells. While high levels of estrogen receptor on neoplastic cells are predictive of a clinically useful response to tamoxifen (1), there have also been reports of more modest activity in neoplasms such as estrogen receptor-negative breast cancers (2), melanomas (16), and desmoid tumors (17), each of which expresses type I IGF receptors but does not have significant concentrations of estrogen receptors.⁴ Our results are consistent with the possibility that the reduction of serum and tissue IGF-I expression is relevant to the activity of tamoxifen in such neoplasms, while the more impressive activity of tamoxifen seen in estrogen receptor-positive neoplasms may involve both this action and various additional mechanisms (1, 18), some of which may be dependent on the expression of estrogen receptors by the neoplastic cells.

This work extends our recent results concerning the effect of tamoxifen on GH release (9, 10), in that we now have evidence not only for hypothalamic-pituitary actions of the drug that result in a reduction of IGF-I levels secondary to the inhibition of GH output, but also for a separate, pituitary-independent mechanism that inhibits IGF-I expression in target tissues for breast cancer metastasis. While it is likely that the former mechanism involves an action of tamoxifen on hypothalamic and/or pituitary estrogen receptors, the molecular mechanism underlying the pituitary-independent action remains to be determined.

The bioactivity of IGF-I within a given tissue is related not only to the degree of expression of the IGF-I gene and to the presence of target type I IGF-I receptors but also to the concentrations of the various IGF-I binding proteins that modulate IGF-I action. It therefore will be of interest to determine the effect of tamoxifen on the expression of these binding proteins. We have recently observed the direct effects of antiestrogens on IGF binding protein expression,⁵ and since previous reports demonstrate that tamoxifen induces transforming growth factor β production (18) and that transforming growth factor β in turn induces IGF-BP3 expression in certain tissues (19), indirect

⁴ M. Pollak *et al.*, unpublished data.

⁵ S. Pratt and M. Pollak, submitted for publication.

pathways by which steroid hormones and their antagonists influence IGF bioactivity may exist as well.

Our data demonstrating an inhibition of IGF-I gene expression by tamoxifen in liver and lung are of interest in the context of the previous demonstration by Murphy *et al.* (20) that estrogen increases rodent uterine IGF-I mRNA levels. Taken together, these results might suggest a simple hypothesis that in general estradiol and tamoxifen have opposite effects on IGF-I expression. However, we believe this interpretation is incomplete, since there is considerable organ-to-organ variability in the estrogenic *versus* antiestrogenic action of tamoxifen. In fact, we have documented that tamoxifen (but not the pure anti-estrogen ICI 182780) increases IGF-I expression in uterus, an observation consistent with the known uterotrophic actions of tamoxifen.⁶

No data are currently available concerning the long-term stability of suppression of IGF-I expression by tamoxifen, and this deserves investigation in the context of the clinically important phenomenon of development of resistance to the antineoplastic effects of tamoxifen over months or years. Further information concerning the contribution to the cytostatic activity of tamoxifen by each of the specific actions of the drug, including the newly described effect on host IGF-I expression reported here, may provide clues to the design of novel, more effective therapies for treatment and prevention of breast cancer and other malignancies.

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