

Induction of Transforming Growth Factor β_1 in Human Breast Cancer *in Vivo* following Tamoxifen Treatment

Anju Butta, Kenneth MacLennan, Kathleen C. Flanders, Nigel P. M. Sacks, Ian Smith, Alan McKinna, Mitchell Dowsett, Lalage M. Wakefield, Michael B. Sporn, Michael Baum, and Anthony A. Colletta¹

The Hartwell Laboratory [A. B., N. P. M. S., M. B., A. A. C.], Section of Academic Surgery and Departments of Histopathology [K. M.], Medicine [I. S.], Surgery [A. M.], and Biochemistry [M. D.], The Institute of Cancer Research and Royal Marsden Hospital, Fulham Road, London SW3 6JJ, United Kingdom, and Laboratory of Chemoprevention, National Cancer Institute, Bethesda, Maryland 20892 [K. C. F., L. M. W., M. B. S.]

Abstract

We have investigated the ability of tamoxifen to regulate members of the transforming growth factor β (TGF- β) family in human breast cancers *in vivo*. Using immunohistochemical techniques, we find that 3 months of tamoxifen treatment causes a consistent induction of extracellular TGF- β_1 in breast cancer biopsies, compared with matched pretreatment samples from the same patient. The induced TGF- β is localized between and around stromal fibroblasts and appears to be derived from these cells. Lower levels of TGF- β_1 , - β_2 , and - β_3 seen in epithelial cells were not altered by tamoxifen treatment. The increased stromal staining of TGF- β_1 occurred in estrogen receptor-negative as well as estrogen receptor-positive tumors. These results provide *in vivo* evidence for a novel, estrogen receptor-independent mechanism of action for tamoxifen, involving the stromal induction of a potent growth inhibitor for epithelial cells.

Introduction

Antiestrogens can be divided into two groups depending on their mechanism of interaction with the ER.² One group of pure antiestrogens such as ICI 164,384 appears to prevent the dimerization of the ER which is essential for transcriptional activation (1). A second group of nonsteroidal antiestrogens such as tamoxifen promotes the efficient binding of the ER to DNA without causing the transcriptional activation seen with full agonists; these are consequently known as partial estrogen agonists (2). The species-, tissue- and cell-specific partial agonist activity of tamoxifen is dependent upon the NH₂-terminal *trans*-activation domain which remains functional in the tamoxifen-ER dimer (3). In addition to these ER-mediated effects, tamoxifen has also been shown to interact with a high affinity microsomal-binding protein of unknown function (4), as well as inhibiting the functions of both calmodulin (5) and protein kinase C (6), but the pharmacological relevance of these observations remains to be established.

Large clinical trials designed to investigate the therapeutic efficacy of adjuvant tamoxifen in the treatment of breast cancer have demonstrated that its advantage is partially independent of the ER content of the primary tumor (7, 8). This indicates that ER expression fails to predict fully any group of patients that might respond to tamoxifen treatment and is consistent with the hypothesis that tamoxifen may have another potent action on tumor growth and not function solely as an antagonist of

estrogen action at the level of the tumor cell. Recently, attention has turned to the interaction of tamoxifen with TGF- β , a family of three multifunctional regulatory peptides which play an important role in controlling proliferation and differentiation in most human epithelial tissues (9). Previous *in vitro* data have shown that tamoxifen can induce autocrine secretion of TGF- β in human breast cancer cells, in which it acts as an inhibitor of growth (10). Tamoxifen has also been shown to induce the secretion of active TGF- β_1 from human fetal fibroblasts despite the demonstrated absence of ER within these cells (11). These fibroblastic cell lines were chosen as an *in vitro* model of breast tumor fibroblasts after the demonstration of a fetal phenotype in fibroblasts isolated from breast tumor specimens (12). Thus, two sets of evidence from *in vitro* studies suggest that tamoxifen might up-regulate the expression of the TGF- β s in breast cancer *in vivo*. Since many breast cancer cell lines retain their responsiveness to this regulatory peptide (13), any TGF- β induced by tamoxifen could potentially play a role in inhibiting tumor cell growth by either autocrine or paracrine mechanisms.

The present study was therefore designed to investigate if tamoxifen regulates TGF- β expression *in vivo* as well as *in vitro*. Immunohistochemical techniques were used to examine the expression of each of the three TGF- β isoforms (9) in matched breast tumor biopsies taken from patients before and after tamoxifen treatment. Polyclonal antibodies raised against peptide epitopes found in the mature region of TGF- β_1 , - β_2 , and - β_3 were used. Our results show a dramatic increase in extracellular TGF- β_1 immunoreactivity after tamoxifen treatment *in vivo* in a series of both ER-positive and -negative tumor samples.

Materials and Methods

Tru-cut biopsy samples were taken before tamoxifen treatment from 10 women with histologically proven invasive ductal carcinomas (5 ER-positive and 5 ER-negative); these women were then placed on a primary medical therapy trial of tamoxifen (20 mg daily) and had another biopsy after 3 months of tamoxifen therapy (14). This brief trial was undertaken to determine if tamoxifen alone would induce a clinical response, prior to surgery. The tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin; sections (4 μ m) were cut and placed on gelatin-coated slides.

For the immunohistochemical localization of TGF- β s (15), sections were permeabilized with 1 mg/ml hyaluronidase in 0.1 M sodium acetate buffer, pH 5.5, containing 0.85% (w/v) sodium chloride solution, and blocked with 1.5% (v/v) normal goat serum in phosphate-buffered saline. The sections were incubated for 2 h at room temperature with the primary polyclonal antibodies to the different TGF- β isoforms at a final concentration of 10 μ g/ml. In a series of preliminary experiments these gentle staining conditions were selected for maximum contrast between the tumor samples taken before and after tamoxifen treatment.

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¹ To whom requests for reprints should be addressed, at The Hartwell Laboratory, Section of Academic Surgery, Institute of Cancer Research, Royal Marsden Hospital, Fulham Road, London, SW3 6JJ, United Kingdom.

² The abbreviations used are: ER, estrogen receptor; TGF- β , transforming growth factor β ; TGF- β_1 -CC, extracellular staining for TGF- β_1 ; TGF- β_1 -LC, intracellular staining for TGF- β_1 .

The TGF- β polyclonal antibodies were raised in rabbits by using synthetic peptides as immunogens and were purified through columns of protein A-Sepharose (anti-TGF- β_1), or the immunizing peptide coupled to Affi-Gel 10 (anti-TGF- β_2 and anti-TGF- β_3). Anti-TGF- β_1 -CC (15) was produced to a peptide corresponding to the first 30 amino acids of mature TGF- β_1 and was obtained from Dr. Larry Ellingsworth, Celtrix Laboratories. This antibody stains extracellular TGF- β_1 but has a slight cross-reactivity with TGF- β_3 on Western blots (16), so the possibility that this antibody may cross-react with TGF- β_3 by immunohistochemistry cannot be excluded. Anti-TGF- β_1 -LC (15) was made to a different synthetic preparation of the identical peptide sequence; it stains intracellular TGF- β_1 . The basis for the differences between the CC and LC antibodies has been investigated in detail and has been confirmed in many studies (15). Anti-TGF- β_2 was raised to a synthetic peptide corresponding to amino acids 50–75 of mature TGF- β_2 (17), and anti-TGF- β_3 was raised to a synthetic peptide corresponding to amino acids 50–60 of mature TGF- β_3 (18). These antisera are specific for the respective TGF- β isoforms, and immunohistochemical staining can be blocked by preincubation with the respective immunizing peptide. The immunohistochemical techniques used recognize latent, as well as active, TGF- β . In the present experiments, normal rabbit IgG at 10 μ g/ml was used as a control. Matched samples from tumors before and after tamoxifen treatment were stained side by side for the various TGF- β isoforms, so as to allow direct comparison of relative staining levels and to minimize the possibility of experimental variation. Bound antibodies were localized with the use of a biotinylated goat anti-rabbit IgG and peroxidase-labeled avidin-biotin complexes from Vector Laboratories. The sections were stained with diaminobenzidine and counterstained with Mayer's hematoxylin (15). Estrogen receptor status in the biopsy samples was determined by the Abbott Laboratories ER Immunocytochemical Assay system.

Results

In tumor samples obtained prior to tamoxifen treatment, use of the anti-CC (extracellular) and anti-LC (intracellular) anti-TGF- β antibodies showed a very weak staining pattern for TGF- β_1 in and around stromal fibroblasts and in epithelial cells (Fig. 1, *a* and *c*; and data not shown). In the present series of experiments, due to the very small amount of experimental material available and the need to maximize the contrast in staining patterns between samples obtained before and after tamoxifen treatment, slides were exposed to the primary antibodies for only 2 h, rather than the 16 h used in previous experiments (15, 16). This resulted in much lower overall staining intensities in the samples obtained from women prior to tamoxifen treatment, compared with the results of an earlier study (16). However, in the present study, a careful examination of the slides revealed staining patterns for TGF- β that were qualitatively similar to those observed previously in a variety of human breast cancers (16).

Following 3 months of tamoxifen treatment a dramatic increase in extracellular TGF- β_1 immunoreactivity was seen in biopsies from all 10 patients (see Table 1). This induced TGF- β_1 is localized predominantly between and around stromal fibroblasts, suggesting these cells as the probable site of synthesis (compare Fig. 1*a* with Fig. 1*b* from a representative ER-positive biopsy, as well as Fig. 1*c* with Fig. 1*d* from a representative ER-negative biopsy). The stromal localization of the site of synthesis is supported by the lack of immunoreactivity with the anti-TGF- β_1 -CC antibody in and directly around the epithelial islands in all the sections studied, and by the presence of immunoreactive intracellular TGF- β_1 in the peritumoral stromal fibroblasts stained with the anti-TGF- β_1 -LC antibody (Fig. 1*e*). After tamoxifen treatment, there was no increase in the weak intracellular TGF- β_1 staining present within the epithelial cells (data not shown). There was, how-

ever, a qualitatively higher posttreatment induction of extracellular TGF- β_1 in the ER-positive biopsies than in the ER-negative biopsies, but with some overlap of staining intensity (see Table 1). As seen in many previous experiments (15, 16), controls stained with normal rabbit IgG showed essentially no immunoreactivity (data not shown).

Our data revealed only weak staining for TGF- β_2 and TGF- β_3 which was localized predominantly in epithelial cells (data not shown), again in agreement with published work (16). However, staining of these isoforms of TGF- β was not changed after 3 months of tamoxifen treatment. Staining for ER in the ER-positive tumor biopsy specimens was confined to the nuclei of epithelial cells, with no staining present in stromal fibroblasts (Fig. 1*f*), in accordance with data from other laboratories (19). As expected, no staining for ER was evident in the ER-negative biopsies (data not shown).

Discussion

We have demonstrated for the first time the regulation of a TGF- β isoform in humans *in vivo* in response to an important pharmacological agent. The striking induction of extracellular TGF- β_1 in the stroma of human breast tumors after 3 months of tamoxifen treatment implicates this peptide growth inhibitor as a potential mediator of the therapeutic action of tamoxifen. This mediation would involve negative paracrine regulation of epithelial cell growth by stromal cells of breast tumors. The induction of TGF- β_1 from stromal cells by tamoxifen supports our earlier *in vitro* observations. In these experiments, tamoxifen was shown to induce the synthesis of active TGF- β_1 by a posttranscriptional mechanism in human fetal fibroblasts, which were used as a model for breast tumor fibroblasts (11).

Our new observations demonstrate a biological action of tamoxifen *in vivo* that is independent of the ER. Our new data suggest that tamoxifen may have significant biological effects via novel pathways, in addition to the antagonism of estradiol at the level of the classical ER. Since the TGF- β s are strongly growth inhibitory for many breast cancer cell lines (13), the present data lend further mechanistic support to the clinical evidence that tamoxifen can be of benefit to patients with ER-negative tumors (7, 8). The qualitatively greater induction of extracellular TGF- β_1 in the ER-positive tumor specimens after tamoxifen treatment may indicate that the epithelial cell ER has an indirect role in the induction, possibly by regulating the production of some soluble factor by the epithelium that modulates the synthesis of TGF- β_1 in stromal fibroblasts, thus leading to a "reciprocal paracrine" effect.

Previous work *in vitro* has shown that tamoxifen, as well as other antiestrogens, can regulate the synthesis and secretion of TGF- β via an ER-dependent mechanism in breast cancer cell lines (10). However, in the present study there was no effect of tamoxifen on epithelial expression of any of the TGF- β s in either ER-positive or ER-negative breast tumors. The discrepancy between the *in vitro* and the *in vivo* data in this instance reinforces the importance of confirming *in vitro* observations with appropriate *in vivo* studies.

Since breast tumor stroma appears to be a main target for the regulation of TGF- β_1 expression by tamoxifen, this raises the possibility that stromal cells may be pharmacologically manipulated to regulate proliferation of malignant breast epithelium through the secretion of paracrine factors. The concept of mesenchymal-epithelial interactions has been previously demonstrated in other hormonally responsive tissues (20). During the period of androgen-stimulated growth and differentiation in the

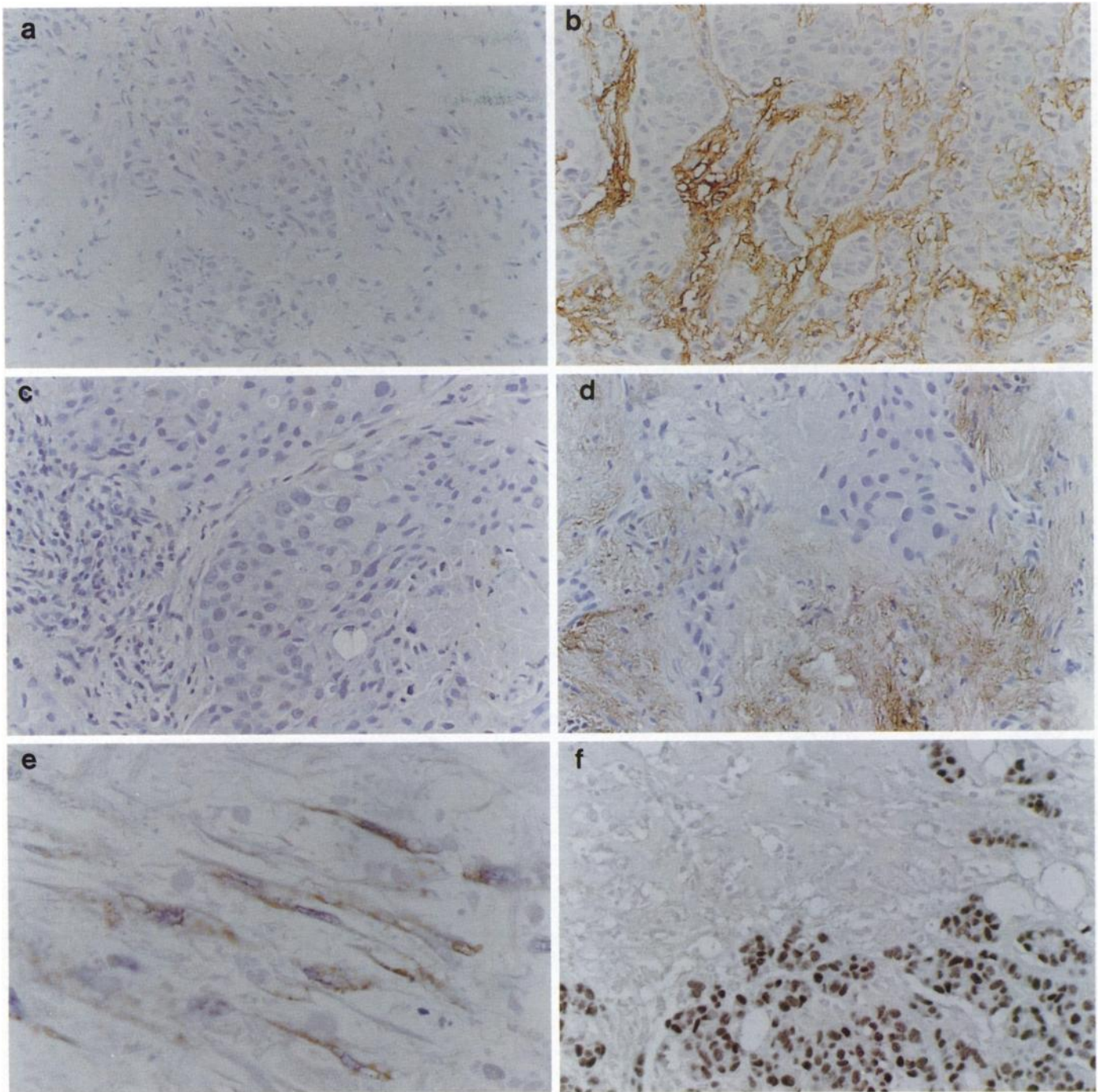


Fig. 1. *a*, ER-positive pretamoxifen treatment biopsy; *b*, ER-positive posttamoxifen biopsy; *c*, ER-negative pretamoxifen biopsy; *d*, ER-negative posttamoxifen biopsy. All four samples were stained with the anti-CC antibody to extracellular TGF- β_1 ; they show a close association of TGF- β_1 with stromal cells and their extracellular matrix. The respective before and after treatment samples were derived from the same tumor and were stained side by side at the same time. Fig. 1*e*, peritumoral fibroblasts, stained with the anti-LC antibody to intracellular TGF- β_1 . Fig. 1*f*, positive staining for ER in a breast tumor biopsy, showing immunoreactivity confined to the nuclei of the epithelial cells with no staining present in the stromal fibroblasts (all magnifications $\times 150$, except *e*, $\times 475$).

prostate gland, the androgen receptor is expressed in mesenchyme prior to its expression in epithelial cells. Elegant studies using tissue recombinants prepared with epithelium and mesenchyme derived from control and androgen-sensitive tissues have shown that androgen-induced development of male accessory organs occurs via soluble factors secreted by mesenchyme (21). These experiments have also shown that androgen-induced epithelial proliferation in the mature prostate is mediated by stromal androgen receptors (21). This supports the idea that certain hormonal effects on epithelium may be elicited indirectly by paracrine influences from its adjacent mesenchyme.

Regulation of TGF- β isoform expression by members of the steroid hormone receptor superfamily *in vivo* is not restricted to stromal elements. An earlier immunohistochemical study demonstrated that all-*trans*-retinoic acid induces TGF- β_2 expression in a wide variety of epithelia in rats (22). Therefore, one possible approach to the chemoprevention of breast cancer might be to use a combination of tamoxifen and retinoic acid or one of its analogues. These could act synergistically to control epithelial proliferation in the breast, in part by enhancing the complementary production of TGF- β_1 by the stroma and TGF- β_2 by the epithelial compartment, during preneoplastic

Table 1 Summary of staining seen in 10 patients' biopsy specimens taken before and after tamoxifen treatment

Slides were assessed independently by two investigators and were assigned staining intensities: -, no staining; \pm , very faint staining; +, weak staining; ++, moderate staining; +++, strong staining. Fig. 1, a and b are taken from patient 1, and Fig. 1, c and d are from patient 7. TGF- β_1 -CC represents the extracellular staining for TGF- β_1 , while TGF- β_1 -LC represents the predominantly intracellular form. Staining for TGF- β_2 and TGF- β_3 was confined to the epithelial components.

Patient	ER	TGF- β_1 -CC		TGF- β_1 -LC		TGF- β_2		TGF- β_3	
		Before	After	Before	After	Before	After	Before	After
1	+	+	+++	++	++	\pm	\pm	\pm	\pm
2	+	+	+++	++	++	-	-	\pm	\pm
3	+	+	+++	++	++	-	-	\pm	\pm
4	+	++	+++	+	++	\pm	\pm	+	+
5	+	+	+++	+	+	\pm	-	+	+
6	-	+	++	+	+	-	-	\pm	\pm
7	-	+	++	++	++	-	-	\pm	\pm
8	-	++	+++	+	+	-	-	+	+
9	-	\pm	++	+	+	\pm	\pm	-	-
10	-	+	+++	++	++	-	-	\pm	\pm

states, before invasive malignancy occurs. In general, it has been found that during the early stages of carcinogenesis epithelial cells retain their sensitivity to the growth-inhibitory actions of TGF- β (13). Thus, if premalignant breast epithelial cells retain responsiveness to TGF- β s, they would then be subject to both autocrine and paracrine regulation by this family of inhibitors. A synergistic interaction between tamoxifen and the retinoic acid analogue, *N*-4-hydroxyphenylretinamide in the prevention of breast cancer has indeed been shown in a rodent model (23).

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