The Growth Inhibition of Human Breast Cancer Cells by a Novel Synthetic Progestin Involves the Induction of Transforming Growth Factor Beta

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Abstract

Recent experimental work has identified a novel intracellular binding site for the synthetic progestin, Gestodene, that appears to be uniquely expressed in human breast cancer cells. Gestodene is shown here to inhibit the growth of human breast cancer cells in a dose-dependent fashion, but has no effect on endocrine-responsive human endometrial cancer cells. Gestodene induced a 90-fold increase in the secretion of transforming growth factor-beta (TGF-beta) by T47D human breast cancer cells. Other synthetic progestins had no effect, indicating that this induction is mediated by the novel Gestodene binding site and not by the conventional progesterone receptor. Furthermore, in four breast cancer cell lines, the extent of induction of TGF-beta correlated with intracellular levels of Gestodene binding site. No induction of TGF-beta was observed with the endometrial cancer line, HECl-B, which lacks the Gestodene binding site, but which expresses high levels of progesterone receptor. The inhibition of growth of T47D cells by Gestodene is partly reversible by a polyclonal antiserum to TGF-beta. These data indicate that the growth-inhibitory action of Gestodene may be mediated in part by an autocrine induction of TGF-beta. (*J. Clin. Invest.* 1991. 87:277–283.) Key words: autocrine • growth factor • breast cancer • progestin

Introduction

The transforming growth factor-beta (TGF-beta)[1] superfamily is composed of many peptides with varying degrees of sequence homology, all of which are involved in the regulation of cell growth and differentiation (1). The prototype member of this family, TGF-beta 1, is a potent inhibitor of the growth of normal epithelial cells (1), and the highly related homologue, TGF-beta 2, appears equally potent in this regard (2). It has been proposed that the loss of sensitivity to growth inhibition by endogenous TGF-betas may contribute to the process of carcinogenesis in some epithelial systems (2). However, many tumor cells retain their responsiveness to growth inhibition by this peptide, raising the possibility that the local induction of TGF-beta could be a useful therapeutic tool (3).

Antiestrogens, such as tamoxifen, have recently been shown to induce the secretion of TGF-beta from human breast cancer cell lines in vitro, suggesting that some of the in vivo effects of this class of drug may be mediated by the induction of such endogenous growth inhibitors (4). This work suggests that other agents might be found that would permit the local manipulation of endogenous levels of different TGF-beta subtypes in a variety of target tissues.

Recently a novel intracellular binding site for a synthetic progestin, that is distinct from any of the classical steroid receptors, has been identified in human breast cancer cells (5). The binding site for this steroidal compound, Gestodene, appears to be expressed uniquely in malignant breast tumor biopsies and cell lines, and not in normal tissues or other malignancies (5, 6). Preliminary studies indicating that Gestodene is growth inhibitory for malignant breast cell lines led us to investigate the possibility that this agent might be acting via the induction of TGF-beta. The data presented here show that growth inhibitory concentrations of Gestodene can induce a large increase in the secretion of TGF-beta types 1 and 2, specifically from malignant breast cells. The implications for the chemoprevention and treatment of breast cancer are discussed.

Methods

Monolayer cell culture and dose-response relationships. All cells were obtained as growing cultures from the American Type Culture Collection and were maintained according to ATCC instructions.

The cells used in this study, together with their maintenance media, are as follows: MCF-7, breast cancer cells, maintained in MEM with 10% FCS, 0.2 iu bovine insulin per ml, 1 mM sodium pyruvate, 2 mM L-glutamine, and nonessential amino acids (NEAA) (all media and additives were obtained from Gibco Laboratories Ltd., UK); T47D, breast cancer cells, maintained in RPMI 1640 medium with 10% FCS, 0.2 iu bovine insulin per ml, and 2 mM L-glutamine; BT-20, breast cancer cells, maintained in MEM with 10% FCS, 1 mM sodium pyruvate, NEAA, and 2 mM L-glutamine; MDA-MB-231, breast cancer cells, maintained in MEM with 10% FCS, and 2 mM L-glutamine; and HEC-1B endometrial cancer cells were maintained in MEM with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and NEAA.

Cells were cultured on Nunclon tissue culture disposables (Gibco Laboratories Ltd.) and were maintained at 37°C in 95% air : 5% CO_2 at 100% relative humidity. The intracellular levels of estrogen and progestin receptors and the Gestodene binding protein in the cells used in this study have recently been reported elsewhere (6).

To assess the effect of Gestodene, compared with a range of control compounds on the growth of breast cancer cells, dose-response relationships in monolayer culture were undertaken. Before these experiments the cells were maintained for 3 wk in phenol red-free media (7) containing 10% FCS from which endogenous steroids had been removed by prior treatment with dextran-coated charcoal (DCC-FCS) (8). Cells were seeded into 25 cm^2 flasks (10^5 cells/flask) in 5 ml of

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1. *Abbreviations used in this paper:* DCC, dextran-coated charcoal; GBS, Gestodene binding site; NEAA, nonessential amino acids; TGF, transforming growth factor.

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phenol red-free medium containing 5% DCC-FCS together with the additives listed above and allowed to attach for 24 h. After this time the seeding medium and any dead cells were removed and replaced with 5 ml of fresh medium. These were then made in quadruplicate, 10, 100, 1000, and 10,000 nM, with respect to the following drugs: dexamethasone, 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione (R5020); 17-alpha-ethinyl-13-beta-ethyl-17-beta-hydroxy-4,15-oestradiene-3-one (Gestodene); 17-alpha-hydroxy-6-methyl-4-pregnane-3,20-dione-17-acetate (MPA); 13-ethyl-11-methylene-18,19-dinorpregn-4-en-20-yn-17-ol-3-one (3-keto-desorgestrel); tamoxifen, 4-chloro-1,2-diphenyl-1-[4-(2-(*N,N*-dimethylamino)ethoxy)phenyl]-1-butene (toremifene) (6). These were all added to the cultures from sterile stock solutions in ethanol, such that the ethanol concentration was at 0.1% vol/vol. Control flasks received 10 nM estradiol alone or ethanol vehicle (0.1% vol/vol) alone. The flasks were then incubated for 8 d. The media and drugs were changed after 48 and 96 h. After the 8-d experimental period, the cells were trypsinized, washed twice with PBS containing 10% DCC-FCS, and counted using a modified Fuchs-Rosenthal hemocytometer (Baird and Toctlock Ltd. London, UK) after viability staining with 0.4% wt/vol trypan blue solution. Results were normalized to the ethanol control cultures which were taken as 100%.

Conditioned media collection and TGF-beta radioreceptor assay. For the preparation of conditioned media, stock cells were grown in phenol red-free media as described above. These were trypsinized and seeded into 175 cm^2 flasks (10^6 cells/flask). The cells were allowed to divide for 6 d, after which time they were ~ 80% confluent. The medium was then removed and the cell monolayers were washed twice with warm PBS. The cells were then incubated for a 24-h period with a defined serum-free medium composed of phenol red-free MEM with 2 mM L-glutamine, 0.2 iu bovine insulin/ml, 5 μg human transferrin/ml, 2 μg bovine fibronectin/ml, 10 ng murine epidermal growth factor/ml, 1 nM sodium selenite, and 0.1 nM cupric sulphate (4), (all obtained from Sigma Chemical Co., St. Louis, MO). After 24 h this medium was discarded and the cell monolayers again washed with warm PBS. The cells were then incubated with 50 ml of the same serum-free medium per flask for a further 48 h (4) with the following drugs in duplicate, estradiol, dexamethasone (both at 10 nM), 3-keto-desorgestrel, levonorgestrol, MPA, R5020, Gestodene, tamoxifen, and toremifene (all at 500 nM). Control flasks contained ethanol vehicle alone at 0.1% vol/vol. After the 48-h incubation the conditioned media were removed to siliconized glass bottles, made 50 μg/ml with ultrapure protease-free bovine serum albumin and 10 μg/ml with aprotinin and leupeptin (Sigma Chemical Co.). The conditioned media were then lyophilized before reconstitution in 5 ml of sterile water. The reconstituted lyophilisates were then dialyzed in Spectrapor 2 dialysis tubing (Pierce-Warriner Ltd. Chester, UK) (molecular weight cutoff 12 kD) against six changes of 50 mM ammonium acetate over a 72-h period at 4°C. The dialyzed conditioned media were then relyophilized in siliconized glass tubes and the total TGF-beta extracted twice with 1 ml aliquots of 4 mM HCl (9). This procedure was necessary to eliminate components that interfered with the radioreceptor assay. These 4 mM HCl extracts were pooled for each individual experimental treatment and clarified by centrifugation at 20,000 *g* for 20 min. The clarified extracts were then used for the TGF-beta radioreceptor assay. The viability of the cells from which the conditioned media were derived was determined.

TGF-beta in the 4 mM HCl extracts from the conditioned media was quantitated in a radioreceptor assay using a two-step format in which the sample and the iodinated ligand are bound sequentially to prevent interference by TGF-beta binding proteins, as described previously (9).

Enzyme-linked immunosorbent assay for TGF-beta 1 and 2. Highly specific sandwich enzyme-linked immunosorbent assays were also used to measure the levels of secreted TGF-beta type 1 and 2 (10). Briefly, Nunc Maxisorb microtiter plates (Roskilde, Denmark) were coated with specific polyclonal rabbit antibodies to TGF-beta 1 or TGF-beta 2. TGF-beta in the conditioned media were then allowed to bind to these adherent antibodies and the resultant immobilized TGF-beta was then detected by secondary turkey anti-TGF-beta type 1 or type 2 antibodies. The sandwiched TGF-betas were then detected colorimetrically by the binding of an alkaline phosphatase-linked goat anti-turkey IgG and incubation with *p*-nitrophenylphosphate.

Immunoprecipitations for TGF-betas 1 and 2. T47D cells were seeded (5×10^5) into 25 cm^2 flasks in MEM containing 5% DCC-FCS and grown for 48 h. The medium was then replaced with cysteine- and methionine-free MEM containing 5% DCC-FCS and 25% of the normal concentration of both cysteine and methionine. To these were added 500 nM tamoxifen, 500 nM Gestodene, or the ethanol vehicle alone (0.1% vol/vol). After 12 h with the drugs, the cells were pulsed for 20 h with [^{35}S]cysteine (0.125 mCi/ml), and the labeled conditioned media were transferred to siliconized tubes and clarified by centrifugation. Aliquots of the clarified supernatants, containing 10^6 trichloroacetic acid-precipitable counts, were then lyophilized after the addition of 10 μg/ml of both phenylmethylsulphonyl fluoride and leupeptin. The lyophilized material was then redissolved in 1 ml of immunoprecipitation buffer (50 mM Tris-HCl pH 7.5, containing 0.15 M NaCl, 1 mM EDTA, 1% Triton-X 100, 1% sodium deoxycholate, 0.1% SDS, and 0.005% thimerosal) and preadsorbed with 100 μg/ml normal rabbit IgG. Samples were then immunoprecipitated with an ammonium sulphate-purified anti-TGF-beta 2 rabbit antibody (S3/28) in duplicate samples, using antibody alone or antibody preincubated for 12 h with 250 ng/ml TGF-beta 2. After precipitation of immunoreactive material with *Staphylococcus aureus,* the supernatants were reincubated with affinity purified rabbit anti-TGF-beta 1 antibody (LC14) either alone, or with antibody preincubated with 250 ng TGF-beta 1, and precipitated as above. The resulting pellets were then washed four times with IP buffer and resolved on a 10% nonreducing SDS polyacrylamide gel according to the method of Laemmli (11). Authentic ^{125}I-TGF-beta was run as a marker.

mRNA extraction and Northern analysis. T47D cells were seeded into 175 cm^2 flasks at 10^7 cells per flask in phenol red-free RPMI containing 5% DCC-FCS and allowed to grow until ~ 50% confluent. At this stage the medium was replaced with fresh medium containing the experimental drugs. The conditions used were the ethanol vehicle control at 0.1% vol/vol, 500 nM Gestodene or 500 nM tamoxifen. These were then grown for a further 48 h and the cells trypsinized and immediately used for RNA preparation by the phenol-chloroform extraction method (12). Total RNA was then poly-A^+ selected by oligo-dT chromatography (12), and 5 μg samples of the resultant mRNA were then resolved on a 1% agarose gel containing formaldehyde and transferred to Genescreen. After prehybridization, the filter was hybridized to a random primer-labeled (13) Sac I/Pvu II fragment derived from the entire porcine TGF-beta 1 cDNA (14). The filter was washed in 0.5× SSC containing 0.1% SDS at 55°C for 30 min and exposed to x-ray film at −70°C for 48 h with intensifying screens. After exposure the filter was stripped, checked for complete removal of probe, prehybridized, and then rehybridized to a random primer-labeled Sph I/Hind III fragment derived from the entire porcine TGF-beta 2 cDNA (15). The filter was then washed as above and exposed to x-ray film at −70°C for 48 h with intensifying screens.

Growth inhibition of T47D cells by TGF-beta and reversal of the growth-inhibitory effect of Gestodene with an anti-TGF-beta antibody. To assess the growth inhibitory activity of TGF-beta types 1 and 2 on breast cancer cells, those cells most strongly growth inhibited by Gestodene (T47D) were tested for their response to the pure growth factors. T47D cells, grown in phenol red-free RPMI with 2% FCS, L-glutamine, and insulin were seeded into 25 cm^2 flasks (5×10^5 cells/flask) in 5 ml of medium and allowed to attach for 24 h. After this time the medium was replaced with 5 ml of fresh medium containing 2% FCS. 100 pM TGF-beta 1, 100 pM TGF-beta 2, or vehicle alone (1% vol/vol 4 mM HCl) were then added to triplicate flasks. These were then cultured for a further 5 d after which time the cells were trypsinized and the viability determined.

To test the reversibility of the growth-inhibitory effect of Gestodene by a polyclonal anti-TGF-beta antibody, 25 cm^2 flasks of T47D cells were set up as above in phenol red-free RPMI with 2% FCS, L-glutamine, and insulin. To these were added in triplicate, 500 nM Gestodene, 500 nM Gestodene plus 50 μg/ml of normal rabbit IgG, and 500 nM Gestodene plus 50 μg/ml affinity purified, rabbit anti-TGF-beta

antibody (R & D Systems, Minneapolis, MN). Control cells received no drug or antibody, and the TGF-beta antibody control received 50 μg/ml of anti-TGF-beta antibody alone. These were allowed to grow for a further 5 d after which time the cells were trypsinized and the viability determined.

Results

Effect of steroids and steroid antagonists on cell growth. The effect of the experimental steroidal agonists and antagonists on the growth of the five cell lines used in this study is illustrated in Fig. 1.

Estradiol causes a significant growth stimulation in all three of the ER + ve cell lines (T47D, MCF-7, and HEC-1B), while having no effect on the two ER-ve lines (MDA-MB-231 and BT-20). All other treatments were either growth inhibitory or had no effect. The antiestrogens, tamoxifen, and its chlorinated derivative, toremifene, were highly growth inhibitory for T47D and MCF-7 breast cancer cells, causing a 20–30% inhibition of cell growth at 1 μM, whereas they had no effect on the ER-ve cells at this concentration. These results are consistent with the antiestrogens exerting their growth inhibitory action through the estrogen receptor. The endometrial cancer cell line HEC-1B, which is ER + ve, showed a slight inhibition by antiestrogens at 1 μM or below and a more substantial inhibition at 10 μM, although effects at this high concentration are not likely to be estrogen receptor mediated (16).

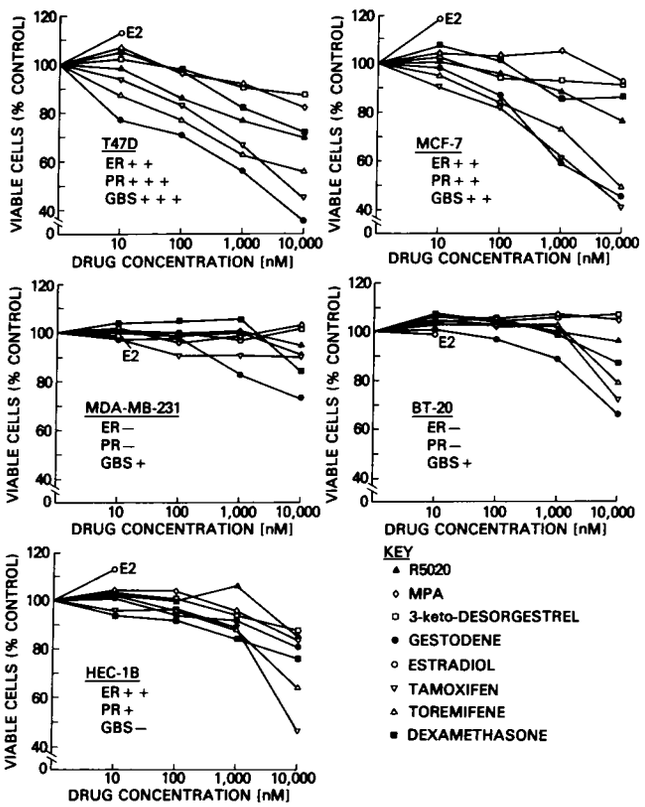

Figure 1. Dose-response relationships of four breast cancer cell lines and an endometrial cancer cell line (HEC-1B) to a range of steroid agonists and antagonists. The data presented here are the means of quadruplicate experiments. The standard deviations are omitted for clarity, but in all instances were < 10% of the mean values. Statistical considerations are discussed in the text. Within the panels: +, < 75 fmol receptor per mg of soluble nuclear protein; ++, > 75 fmol receptor per mg of soluble nuclear protein; +++, > 140 fmol receptor per mg of soluble nuclear protein. (For a full discussion see reference 6.)

The novel synthetic progestin, Gestodene, caused a dose-dependent inhibition of the growth of all four breast cancer cell lines with significant inhibition of growth at less than 1 μM. Growth inhibition was greatest (>40% inhibition of growth at 1 μM) in the T47D line which expressed the highest levels of the Gestodene binding site (GBS). The degree of inhibition of the other three breast cancer cell lines correlated well with the level of GBS expression. For the T47D cells, Gestodene was significantly more growth inhibitory than the antiestrogens, and in the MCF-7 cells, Gestodene was as active as tamoxifen and significantly more active than toremifene. Since Gestodene was also growth inhibitory for the two ER-ve breast cancer cell lines, where the antiestrogens had no effect, this indicates that Gestodene has a wider range of action than the antiestrogens.

The growth inhibitory action of Gestodene was independent of the classical progesterone receptor. Thus, both the MDA-MB-231 and BT-20 cell lines, which contain no progesterone receptor (17), were inhibited by Gestodene at 1 μM or above. Conversely, Gestodene had no effect on the HEC-1B cell line, which expresses progesterone receptor but contains no GBS. The synthetic progestins MPA and 3-keto-desorgestrel showed very little or no inhibitory effect on any of the cell lines, consistent with the interpretation that Gestodene is causing growth inhibition through the GBS and not the progesterone receptor. Kruskal-Wallis statistical analysis of these data reveal that Gestodene is significantly ($P < 0.05$) more active in inhibiting the growth of the four breast cancer cell lines than any of the other compounds tested. Due to the cross-receptor binding of Gestodene that will inevitably occur with the progesterone receptor positive T47D and MCF-7 cells, it is not possible to attribute the activity of this compound solely to its own intracellular binding site, although the withdrawal of all estrogens from these cells for a period of time before the experiments was designed to reduce progesterone receptor levels and thus minimize interference from progesterone receptor-mediated events (7). When the cells are ranked in decreasing intracellular concentration of the Gestodene binding site (T47D, MCF-7, MDA-MB-231, and BT-20), there is a parallel increase in the number of surviving, viable cells over the time course of the experiment. Thus at 1,000 nM Gestodene the number of surviving cells increases from 56% (T47D) to 60% (MCF-7), 85% (MDA-MB-231), and 90% (BT-20). This provides indirect evidence at least that the growth inhibitory activity of Gestodene in these experiments is related to the novel binding protein.

Quantitation of secreted TGF-beta by radioreceptor assay. The results of the radioreceptor assays on the conditioned media produced by the breast cancer cells in response to the experimental drugs are shown in Table I. The data are presented as the total TGF-beta secretion rates in ng/10^6 cells per 48 h, with the fold induction normalized to the vehicle control for each individual cell line. The four breast cancer cell lines all show an induction of TGF-beta levels above vehicle control levels after treatment with Gestodene. These inductions vary from 94-fold for the T47D cells ($P < 0.001$) to 3-fold for the BT-20 cells ($P < 0.05$) over vehicle alone controls. Induction of TGF-beta by tamoxifen appears to be mediated through the conventional estrogen receptor as inductions of 32-fold (T47D; $P < 0.001$ versus control) and 10-fold (MCF-7; $P < 0.001$ versus control)

Table I. Total TGF-beta Secretion Rates±Standard Deviations (n = 4) for the Range of Breast Cancer Cell Lines Used in This Study, Together with the Endometrial Cancer Line HEC-1B, as Determined by the Radioreceptor Assay

Cell type	Treatment	TGF-beta secretion rate	Fold induction
		ng/10⁶ cells/48 h	
T47D	Control	<0.2	"1"
	E2	<0.2	1
	Tamoxifen	6.3±0.7	32
	Gestodene	18.8±3.4	94
MCF-7	Control	0.6±0.2	"1"
	E2	0.5±0.1	1
	Tamoxifen	2.4±0.3	4
	Gestodene	5.6±1.7	10
MDA-MB-231	Control	0.4±0.1	"1"
	E2	0.4±0.1	1
	Tamoxifen	0.5±0.2	1
	Gestodene	2.6±0.5	6
BT-20	Control	0.3±0.1	"1"
	E2	<0.2	1
	Tamoxifen	0.3±0.1	1
	Gestodene	0.9±0.1	3
HEC-1B	Control	0.2±0.1	"1"
	E2	0.2±0.1	1
	Tamoxifen	<0.2	1
	Gestodene	0.2±0.1	1

The drug concentrations used are given in Methods. The fold inductions are normalized to the control value for each cell line. Statistical values are given in Results.

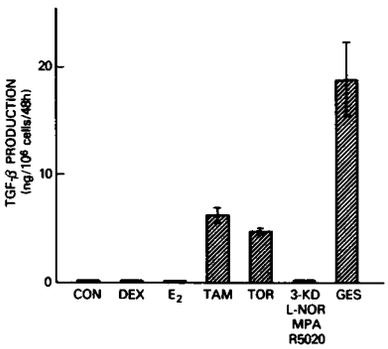

Figure 2. Total TGF-beta secretion by the T47D breast cancer cell line, in response to the complete range of compounds used for the dose-response curves illustrated in Fig. 1, as measured by the radioreceptor assay. The concentrations of the drugs are given in Methods. The data presented are the means±SD (n = 4). Statistical considerations are discussed in the text.

are only seen in those cells that contain estrogen receptors. Estradiol had no effect on the synthesis of TGF-beta by any of these cells. The HEC-1B endometrial carcinoma cell line, which possesses both estrogen and progesterone receptors, and which is only growth inhibited by tamoxifen at cytotoxic concentrations (Fig. 1), showed no increase in TGF-beta secretion to either tamoxifen or Gestodene. As the Gestodene binding protein is not expressed in this cell line, this confirms that the induction of TGF-beta by Gestodene is not mediated by the conventional progesterone receptor.

The secretion of TGF-beta in the T47D cell line was characterized in response to the full range of compounds used in the dose-response experiments illustrated in Fig. 1. These data are shown in Fig. 2. The ethanol control levels of secretion are essentially identical to those seen in T47D cells treated with 10 nM estradiol, 10 nM dexamethasone, or 500 nM of the synthetic progestins, 3-keto-desorgestrel, levonorgestrel, MPA, and R5020. Both tamoxifen and toremifene cause 25- to 30-fold inductions over the control levels ($P < 0.001$ versus control), whereas Gestodene causes a ~ 90-fold induction over the control levels ($P < 0.001$ versus both the control and the tamoxifen/toremifene levels). The TGF-beta secreted by the majority of cells in culture is in a biologically latent form that can be activated in vitro by transient acidification or heating (9, 22). To determine the fraction of TGF-beta secreted by T47D cells in the biologically active state, conditioned media from these cells was assayed directly in the radioreceptor assay without the dialysis and acid extraction steps that were otherwise used. The results are shown in Table II. Significant receptor binding activity, that was neutralized by anti-TGF-beta antibodies, was present in the unprocessed conditioned medium, suggesting the presence of active TGF-beta. Both the antiserum and the TGF-beta receptor recognize the active form of TGF-beta alone (9). Neither heating the conditioned media to 80°C for 5 min, nor transient acidification to pH2 caused any real increase in receptor-reactive TGF-beta, indicating that the conditioned media did not contain any latent TGF-beta. Thus, essentially all the TGF-beta secreted by T47D cells in response to Gestodene is in the biologically active form. The inability of the anti-TGF-beta antibodies to completely reverse the receptor-binding activity in the conditioned medium appears to be due to components that interfere in the binding assay. For accurate determination of total TGF-beta levels (Fig. 2 and Table I) these components were removed by dialysis and acid extraction of the conditioned media.

Table II. Lack of Effect of Heat or Acid Activation on TGF-beta Receptor-Competing Activity in Conditioned Medium Collected from T47D Cells Treated with 500 nM Gestodene

Sample	% Competition in radioreceptor assay
50 pM TGF-beta	50
Conditioned medium: untreated	52
+70 mg/ml normal rabbit IgG	50
+70 mg/ml anti-TGF-beta IgG	29
Heated 5 min. at 80°C	50
Acidification to pH2	39
Premixed acid/base control	41

The conditioned medium was diluted 2-fold into PBS containing 0.01% BSA and assayed directly for ability to compete with ^{125}I-labelled TGF-beta for binding to the TGF-beta receptor on A541 cells. Parallel samples were either heated to 80°C for 5 min or transiently acidified to pH2 with 5 M HCl and reneutralized with 5 M NaOH, 1 M Hepes in order to activate any latent TGF-beta that might be present before the assay. Interfering components in the conditioned medium account for the residual competing activity that is not neutralized by anti-TGF-beta antibodies. Results are the means of three determinations, standard deviations were < 5% of the mean values.

Subtype analysis by enzyme-linked immunosorbent assay for TGF-beta 1 and 2. The TGF-beta subtype analysis and total TGF-beta concentrations in conditioned media from T47D cells, as determined by enzyme-linked immunoassay, are presented in Table III. These data show that the majority (~ 90–95%) of the TGF-beta secreted by these cells is type 1 with a small component (5–10%) of type 2. The total TGF-beta levels are approximately equivalent to those measured by the radioreceptor assay, but the fold induction by the Gestodene treated T47D cells over the vehicle control is 36-fold, somewhat less than seen with the radioreceptor assay. This appears to be due to the higher level of TGF-beta detected in the control conditioned media when measured with the immunoassay technique rather than the radioreceptor assay.

Immunoprecipitation of TGF-beta 1 and TGF-beta 2. Autoradiographs of SDS-polyacrylamide gels which were used to analyze the metabolically-labeled, immunoprecipitated TGF-beta 1 and 2 from T47D breast cancer cells are illustrated in Fig. 3. The use of antibodies with or without prior incubation with large amounts of purified TGF-beta 1 or 2 illustrates the specificity of these reagents. The upper panel in Fig. 3 shows that the control T47D cells constitutively secrete a small amount of TGF-beta 1. The next lane shows that this secretion is dramatically increased after treatment of these cells with 500 nM Gestodene. The effect with 500 nM tamoxifen is far less pronounced than the Gestodene effect but is still greater than that seen with the ethanol vehicle alone. There is also some increase in the amount of TGF-beta 2 produced by these cells in response to both of the drugs, as seen in the lower panel of Fig. 3.

Thus, there is an increase in the de novo synthesis of TGF-beta 1 and 2 in response to both Gestodene and tamoxifen in the T47D breast cancer cells.

Northern analysis of TGF-beta 1 mRNA. Northern blot analysis shows a small (2- to 3-fold) increase in TGF-beta 1 mRNA in T47D cells after the 48-h treatment with 500 nM Gestodene (Fig. 4). There does not appear to be any increase in TGF-beta 1 message after treatment with tamoxifen, as previously reported for the MCF-7 breast cancer cell line (4). Thus, only a fraction of the large increase in the secretion of TGF-beta 1 by T47D cells in response to Gestodene is due to transcriptional regulation of the mRNA for this protein.

Growth inhibition of T47D cells by TGF-beta and antibody reversal of the growth inhibitory effect of Gestodene. To test the hypothesis that the growth inhibitory effect of Gestodene on the T47D cell line is mediated through the induction of the TGF-betas, the pure peptides were examined for their effects on the growth of this cell line.

Table III. Subtype Analysis and Total TGF-beta Secretion by the T47D Breast Cancer Cells, as Determined by the Sandwich ELISA

Treatment	TGF-beta 1	TGF-beta 2	TGF-beta 1
			% of Total
Control	0.50±0.10	0.08±0.0	86
E2	0.10±0.0	<0.20	ND
MPA	0.30±0.0	0.07±0.0	92
Toremifene	5.60±0.20	0.51±0.10	94
Tamoxifen	6.30±0.30	0.65±0.10	91
Gestodene	20.20±2.90	1.20±0.10	91

The drug concentrations are given in Methods. The TGF-beta levels are in ng/10^6 T47D cells per 48 h in serum-free culture. Values are means±SD (n = 4).

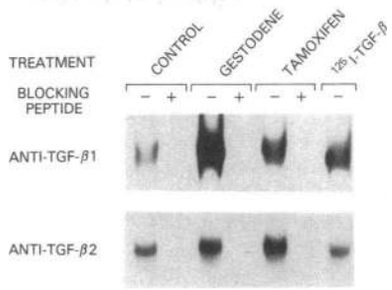

Figure 3. Autoradiographs of SDS polyacrylamide gels used to analyze the anti-TGF-beta immunoprecipitation products from drug-treated T47D cells metabolically labeled with [^{35}S]methionine. Aliquots of metabolically labeled whole cell extracts were immunoprecipitated with a rabbit anti-TGF-beta 2 antibody, using antibody alone (−) or antibody preincubated for 12 h with 250 ng/ml TGF-beta 2 (+). After removal of antibody complexes, the extracts were further incubated with anti-TGF-beta 1 antibody, again using antibody alone (−) or antibody preincubated with 250 ng/ml TGF-beta 1. 10,000 cpm of ^{125}I-TGF-beta 1 or 2 were also immunoprecipitated as markers.

After 5 d in culture with 100 pM TGF-beta 1 the T47D cells were inhibited by 19±4% compared with vehicle controls ($P < 0.05$). 100 pM TGF-beta 2 caused a much greater inhibition of these cells, by 43±8% compared with vehicle controls ($P < 0.01$). These data contrast with a recent report that claimed that the T47D cell line was unaffected by such concentrations of TGF-beta, a discrepancy that might reflect the phenotypic drift of these cells.

The data in Fig. 5 show that the inhibition of T47D cell growth by Gestodene can be partly reversed by co-treatment with 50 μg/ml of a polyclonal antiserum that neutralizes both TGF-beta 1 and 2. Anti-TGF-beta antiserum alone had no intrinsic effect on the growth of these cells, and normal rabbit IgG did not reverse the growth inhibitory effect of Gestodene. Anti-TGF-beta antiserum reverses the growth inhibition from 36±18% to 76±7% of control cell numbers ($P < 0.01$ versus Gestodene plus normal rabbit IgG). These data provide good evidence that the growth inhibition of T47D cells by this novel steroidal compound is at least partly mediated by the autocrine induction of the TGF-betas.

Discussion

The data presented here demonstrate that the novel progestational agent, Gestodene, is a potent inhibitor of the growth of

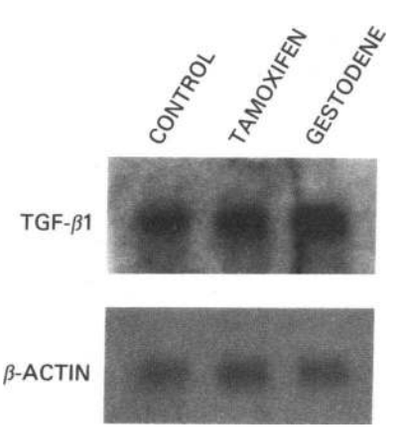

Figure 4. Northern analysis of mRNA from drug-treated T47D cells by hybridization to the TGF-beta 1 cDNA. 5-μg samples of mRNA were fractionated on a 1% agarose gel containing formaldehyde. After transfer this was probed with the TGF-beta 1 probe (*above*) and then reprobed with a B-actin probe to determine loading equivalence (*below*).

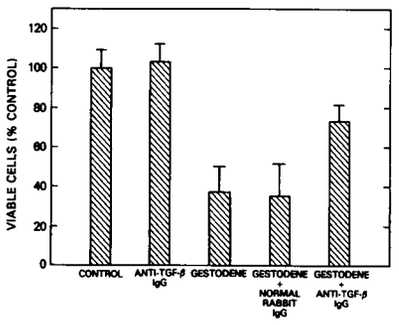

Figure 5. Partial reversal of the growth-inhibitory effect of Gestodene on T47D cells with an anti-TGF-beta antibody. T47D cells received either ethanol vehicle alone, 500 nM Gestodene, 500 nM Gestodene plus 50 μg/ml normal rabbit IgG, or 500 nM Gestodene plus 50 μg/ml rabbit anti-TGF-beta antibody. The viable cells remaining after 5 d are expressed as a percentage of the ethanol controls, and are the means of triplicate experiments±the standard deviations.

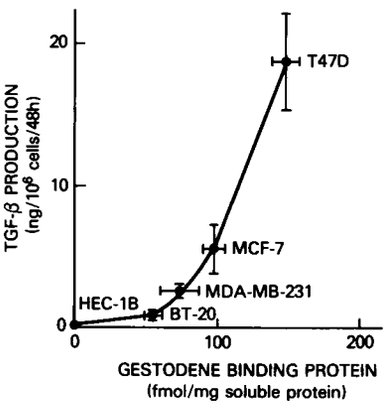

Figure 6. Relationship between the intracellular Gestodene binding protein (fmol per mg of soluble cellular protein) content of the four breast cancer cell lines used in this study and the HEC-1B cell line, and the total TGF-beta secretion (ng/10^6 cells per 48 h) by these cells in response to 500 nM Gestodene. Data are presented as the means of quadruplicate measurements with the standard deviations presented in both orientations.

breast cancer cells in vitro. Furthermore, the growth inhibitory effect of Gestodene on these cells appears to be mediated at least in part by the induction of a negative autocrine loop involving TGF-beta 1. The identification of a pharmacological agent that induces the secretion of a potent endogenous inhibitor of cell growth, specifically in breast cancer cells, has implications for the therapy and chemoprevention of breast cancer, as discussed below.

In all four breast cancer cell lines examined here, Gestodene was at least as potent in inhibiting cell growth as the antiestrogen tamoxifen which is currently in clinical use.

Growth inhibition by Gestodene does not involve the classical progesterone receptor, but appears to be mediated by a novel high affinity intracellular binding site that has recently been identified uniquely in breast cancer cells and tissues (5, 6). Thus the degree of inhibition by Gestodene correlated well with levels of GBS. Other synthetic progestins showed no growth inhibitory effects on these cells. The tissue specificity of Gestodene is reinforced by the observation that the endometrial cancer cell line, HEC-1B, which expresses progesterone receptor but not GBS, was entirely unaffected by Gestodene.

The growth inhibition seen with Gestodene was >60% reversed by the addition of specific antibodies against TGF-beta, suggesting that a major component of the growth inhibition was due to the induction of TGF-beta, a potent inhibitor of epithelial cell growth (2). The correlation between the levels of intracellular GBS in the breast cancer cells and the amount of TGF-beta secreted in response to Gestodene treatment (Fig. 6), suggests the importance of the GBS in this induction.

The increase in the levels of TGF-beta 1 secreted by the Gestodene-treated T47D cells was at least one order of magnitude greater than the observed increase in the TGF-beta 1 mRNA levels. This suggests that the mechanism of induction of TGF-beta 1 by Gestodene involves effects predominantly at a posttranscriptional level. Thus Gestodene might effect the efficiency of translation of the TGF-beta 1 mRNA or the post-translational processing, routing, or stability of the TGF-beta 1 protein. The induction of TGF-beta by antiestrogens in the MCF-7 cell line (4) and in human fetal fibroblasts that lack the estrogen receptor (18), appears to involve similar posttranscriptional mechanisms.

The TGF-beta secreted by the breast cancer cells in response to Gestodene appeared to be predominantly in the biologically active rather than the more common latent form. All the conditioned media samples had to be acid extracted or precipitated for the accurate quantitation of TGF-beta levels by the radioreceptor or ELISA assays, a procedure that activates all latent TGF-beta (9). However, the ability of neutralizing antisera that recognize only the active form of TGF-beta to partially reverse the growth inhibitory effect of Gestodene on the T47D cells indicated that the cells were secreting active TGF-beta. Furthermore, the inability of transient acidification or heat treatment to increase the assayable TGF-beta in unprocessed conditioned medium confirmed that essentially all the TGF-beta secreted was in the active, not latent form. The TGF-beta secreted by MCF-7 cells and by human fetal fibroblasts was also partly in the active form (4, 18), as was the TGF-beta 2 secreted by keratinocytes in response to retinoids (19). Thus it may be a common feature of the steroid hormone family to induce TGF-beta in an active form. This has important physiological consequences, since the ubiquitous presence of cell-surface binding proteins for TGF-beta (9) and the rapid clearance of active TGF-beta from plasma (20) probably ensure that any TGF-beta secreted in the active form can only act on target cells very close to the site of production. While the target cells for latent TGF-beta have yet to be identified, all cells that express the TGF-beta receptor are potential targets for active TGF-beta.

The finding that the Gestodene binding site is uniquely expressed in breast cancer cells (6) may thus provide a novel approach for the treatment of breast cancer. In normal breast epithelial cells, Gestodene would act only as a progestin, but in malignant cells the presence of the GBS would ensure the synthesis of TGF-beta, which would then act in an autocrine and paracrine fashion to inhibit cancer cell growth. Tissue-specific inducers of active TGF-beta, such as Gestodene, may thus allow the targeting and highly localized induction of this powerful growth inhibitor. Given the pleiotropic activities of TGF-beta, experiments in vivo are now required to see whether the beneficial effects of TGF-beta in the inhibition of malignant cell growth will outweigh the potentially serious effects of promoting stromal development and inhibiting immune surveillance (2, 21).

As Gestodene is a high affinity progestin (6) it may also offer potential as a chemopreventive agent for breast cancer. Offered as part of a combined oral contraceptive preparation, it would have no effect on the breast epithelium as long as the cells were dividing normally. Any cells that became malignant

and thus start to express the GBS would then respond to Gestodene by the secretion of elevated levels of TGF-beta. This would then lead to the growth inhibition of these cells. This interventional approach to chemoprevention depends on the precise stage in the carcinogenic process at which these cells begin to express the GBS. This remains unknown at present but current efforts to purify the Gestodene binding protein should provide reagents which will allow the study of this event.

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