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Absence of Transforming Growth Factor-β Responsiveness in the Tamoxifen Growth-Inhibited Human Breast Cancer Cell Line CAMA-1

Hongjun Ji, Laurence E. Stout, Qingqing Zhang, Ruoping Zhang, Helen T. Leung, and Benjamin S. Leung

Department of Obstetrics and Gynecology (H.J., L.E.S., Q.Z., R.Z., H.T.L., B.S.L.) and Department of Animal Physiology (H.J., Q.Z., B.S.L.), University of Minnesota, Minneapolis, Minnesota 55455

Abstract Tamoxifen has been an effective antiestrogen in suppressing breast cancer growth which is estrogen-responsive or dependent. Early studies have provided circumstantial evidence that transforming growth factor-β (TGF-β) may be an autocrine mediator of tamoxifen action. Therefore, it is both fundamentally important and clinically relevant to investigate the relationship between tamoxifen and TGF-β. In this study, we demonstrated that CAMA-1 cells, which are sensitive to tamoxifen inhibition, did not respond to TGF-β growth inhibition. The type I and II TGF-β receptors were undetectable by the radio-ligand affinity labeling technique. Despite the presence of a normal TGF-β type II receptor gene, the mRNA transcript of the gene was undetectable by the extremely sensitive Intron-differential RNA/PCR method. The possibility that the lack of TGF-β receptors might be intimately linked to the absence of normal retinoblastoma (Rb) gene products, as suggested by previous studies of retinoblastoma cells, was further investigated. The lack of TGF-β receptor expression was found due to reasons other than the absence, deletion or abnormality of the Rb gene because a normal Rb gene and its hyper- and hypo-phosphorylated protein products were detected in CAMA-1 cells. In conclusion, our results suggest that the TGF-β system is not obligatory for antiestrogen growth inhibition of CAMA-1 cells.

Key words: TGF-β receptor, human TGF-β type II receptor, retinoblastoma gene, retinoblastoma gene products, intron-differential RNA/PCR

INTRODUCTION

The hypothesis that growth factors may serve as autocrine factors to stimulate tumor cell proliferation has been well documented since its inception [Sporn and Todaro, 1980]; this hypothesis has now been extended to include negatively regulated cell growth by auto-inhibitory factors [Sporn and Roberts, 1985]. The important role of growth factors in tumorigenesis is widely accepted [Goustin et al., 1986]. The positive and negative homeostatic regulation of cell growth in normal tissues is believed to be altered in transformed and tumor cells. An imbalance between activation of growth-stimulatory factors and inactivation of growth-inhibitory factors can result in a surfeit of growth stimulation, a deficient growth inhibition of tumor cells, or a combination of both conditions. Conceivably, intervention of the activation or inactivation process of these growth modulating factors may have therapeutic potentials in the treatment of cancers.

Growth factors regulate growth and differentiation by triggering the signal transduction mechanism via binding to their cognate membrane receptors. For transforming growth factor-β (TGF-β) proteins, which belong to a multifunctional family of growth and differentiation factors [Massague, 1990; Roberts and Sporn, 1990], there are three distinct membrane receptors (named as type I, II, and III receptor) as determined by affinity ligand radiolabeling technique [Boyd et al., 1990]. Both the type II receptor, which contains a transmembrane serine/threonine kinase [Lin et al., 1992], and the type

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Qingqing Zhang's present address is Human Genome Sciences, Inc., 9620 Medical Center Drive, Rockville, MD 20850-3338.

Address reprint requests to Dr. Benjamin S. Leung, Department of Obstetrics and Gynecology, University of Minnesota, Minneapolis, MN 55455.

III receptor, which is a betaglycan with a core protein of 853 amino acids [Wang et al., 1991], have recently been cloned. Type I and II receptors are essential for TGF-β growth inhibition of epithelial cells [Boyd and Massague, 1989; Geiser et al., 1992].

The effects of growth factors on breast cancer cell proliferation have been intensely studied in recent years, especially in understanding their relationship with steroid hormone actions [Lippman et al., 1987, 1988; Dickson and Lippman, 1988; Lippman and Dickson, 1989; Cullen et al., 1989]. In particular, TGF-α (TGF-α) has been implicated in mediating estrogen-induced proliferation and TGF-β, in mediating antiestrogen inhibition of breast cancer cells [Dickson et al., 1986; Lippman et al., 1986; Knabbe et al., 1987]. This hypothesis is best summarized in the paper by Cullen et al. [1989]: (1) estrogen is important in the genesis of breast cancer, (2) numerous estrogen-regulated growth factors can act in an autocrine or a paracrine fashion to control the growth of estrogen-dependent tumors, (3) these same growth factors can be produced in a constitutive or unregulated manner in the estrogen-nonresponsive tumors, and (4) antiestrogen inhibition of breast cancer growth can be mediated by down-regulation of specific growth-stimulatory growth factors as well as by up-regulation of specific growth-inhibitory growth factors. However, several recent studies [Arteaga et al., 1988a; Osborne et al., 1988; Clarke et al., 1989; Arteaga and Osborne, 1990] have questioned the validity of TGF-α in mediating estrogen action. We have previously demonstrated that estrogen-regulated cell proliferation in human breast cancer CAMA-1 cells is independent of the epidermal growth factor (EGF)/TGF-α pathway [Leung et al., 1991]. In this report, we present compelling evidence that antiestrogen inhibition of CAMA-1 cell proliferation does not involve TGF-β action. Our results show that CAMA-1 cells are insensitive to growth inhibition of TGF-β due to the lack of functional TGF-β type I and II receptors but remain sensitive to tamoxifen inhibition.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's Medium/F12 (DMEM/F12) medium was obtained from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD). Fetal bovine serum (FBS) was obtained from HyClone Laboratories, Inc. (Logan, UT). Serum substitute (SS) was prepared as previously described [Leung, 1987]. Transforming growth factor-β1 (TGF-β1), purified from human platelets, was a gift from C. Heldin (Uppsala, Sweden). λDNA/*Hin*dIII marker, SDS-6H high-molecular-weight (MW) standard mixture, and SDS-7B prestained SDS MW standard mixture were bought from Sigma Chemical Company (St. Louis, MO). Monoclonal antibody (Ab-4) against retinoblastoma proteins was obtained from Oncogene Science, Inc. (Uniondale, NY). Protein A–agarose conjugate (IPA-300, Immobilized rProtein A) was purchased from Repligen Corporation (Cambridge, MA). GeneScreen Plus nylon membrane was obtained from E. I. du Pont de Nemours & Co., (Inc.), NEN Products (Boston, MA). Nitrocellulose membrane was from Schleicher and Schuell, Inc., Keene, NH. Alpha-[^{32}P]dATP (6,000 Ci/mmol) was purchased from Amersham Corporation (Arlington Heights, IL). Radiolabeled ^{125}I-TGF-β1 was a gift from Collaborative Biomedical Products, Becton Dickinson Labware (Bedford, MA). Oligonucleotides were synthesized on an Applied Biosystem 391 PCR-MATE DNA synthesizer.

Cell Cultures

Cell lines of breast cancer (MCF-7_{NIH} from National Institute of Health, MCF-7_{MI} from Michigan Cancer Foundation, and CAMA-1) and ovarian cancer (CAOV-3 and NIH-OVCAR-3) were routinely cultured in monolayers in DMEM/F12 media with 10 to 15% FBS and 100 units/ml penicillin + 100 μg/ml streptomycin. CAMA-1 cells were normally cultured in phenol-red media supplemented with 1 nM of 17β-estradiol, and MCF-7 and OVCAR-3 supplemented with 10 μg/ml of insulin.

Cell Growth Curve of CAMA-1 in the Presence of TGF-β1

For investigating the effect of TGF-β on cell proliferation, CAMA-1 cells were partially synchronized for 48 hours without serum prior to plating in 12-well cluster plates (Costar). To each well 5% FBS, 1 nM 17β-estradiol and various concentrations of TGF-β1 were added. The medium was changed and TGF-β1 was added fresh every other day during the growth curve. Plasticware was siliconized and FBS was used in dilution of TGF-β1. Cells from replicate plates were trypsinized and cell counts were deter-

mined by a Coulter Counter, model ZBI (Coulter Electronics, Inc., Hialeah, FL).

Affinity Radioligand Binding

Experiments to determine TGF-β receptors by affinity ligand binding with ^{125}I-TGF-β1 were conducted according to the method of Massague and co-workers [Massague and Like, 1985; Cheifetz et al., 1986; Massague, 1987].

Intron-Differential RNA/PCR

Detection of human TGF-β type II receptor gene [Lin et al., 1992] expression by the intron-differential (ID) RNA/PCR was conducted as previously described [Ji et al., 1990; Leung et al., 1991]. A 120 base-pair (bp) cDNA PCR product is expected from the up-stream sense Primer 1 (5′-ATGGGTCGGGGGCTGCTCAG-3′) and down-stream antisense Primer 2 (5′-GTCAGTGACTATCATGTCGT-3′). The cDNA product (120 bp) was detected by an internal oligonucleotide probe (5′-CCGACTTCTGAACGTGCGGT-3′) in Southern analysis. Forty-five cycles of denaturing (94°C, 15 sec), annealing (55°C, 15 sec), and extension (72°C, 15 sec), with an additional 1 min incubation at 72°C after the completion of the last cycle, were performed on a Twin-Block Thermocycler (Ericomp Inc., San Diego, CA). Primers and Probe for human c-*myc* ID RNA/PCR were as described previously [Ji et al., 1990].

Southern Blotting

High-molecular-weight genomic DNA (10 μg), was isolated as described (Ausubel et al., 1989), digested with excess endonucleases such as BamHI, EcoRI, and Hind III, electrophoretically fractionated on 1% agarose, and transferred onto GeneScreen Plus nylon membrane using a Bio-Rad vacuum blotter (model 785). The nylon filter was baked at 80°C under vacuum for 30–60 min and UV crosslinked for 20 secs. Prehybridization (1 h) and hybridization (20–24 h) were carried out at 65°C in solution containing 6 × SSC, 5 × Denhardt's reagent, 0.5% sodium dodecyl sulfate (SDS), and 100 μg/ml denatured sonicated salmon sperm DNA [Sambrook et al., 1989]. The 4.5 kb *Eco*R1 fragment from H2-3FF [Lin et al., 1992] was used to prepare the human TGF-β type II receptor probe and the 0.9 kb and 3.8 kb *Eco*R1 fragments from pGH2 and pG4 [T'Ang et al., 1988], respectively, were used as human retinoblastoma (Rb) probes. The probes were radiolabeled with α-[^{32}P]dATP by a random-priming procedure [Feinberg and Vogelstein, 1983]. Washing was carried out twice for 15 min at room temperature in 2 × SSC-0.1% SDS and once for 20 min at 65°C in 0.2 × SSC-0.1% SDS. The filter was exposed to an X-ray film with intensifying screens at −70°C.

Western Blotting

Cells grown in 100 mm dishes were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with ice-cold EBC lysis buffer [50 mM Tris–HCl (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM Na orthovanadate, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 4 mM EDTA]. The cellular debris was pelleted by centrifugation at 4°C for 30 min in a microcentrifuge (Fisher MICRO-CENTRIFUGE, model 235A, Fisher Scientific, Itasca, IL). One μg of anti-pRB monoclonal antibody (Ab-4) was added to the EBC lysate and incubated at 4°C for 1 h, and the complex was immunoprecipitated with 15 μl of Protein A–agarose at 4°C for 1 h with constant agitation. The immunoprecipitate was pelleted by 2-min centrifugation in a low-speed microcentrifuge (Capsule HF-120, Tomy Seiko, Tokyo, Japan), washed twice with EBC lysis buffer, and dissolved in 15 μl of EBC and 15 μl of 2 × sample buffer [125 mM Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% β-mercaptoethanol]. The samples were electrophoretically resolved on a 7.5% SDS-polyacrylamide gel [Laemmli, 1970] and blotted overnight onto a 0.45-μm nitrocellulose membrane [Towbin et al., 1979]. After blocking with 2% BSA (bovine serum albumin fraction V from Sigma), the membrane was incubated with the anti-pRB monoclonal antibody (Ab-4), probed with biotinylated secondary antibody and streptavadin-alkaline phosphatase-conjugate solutions (Bio-Rad Laboratories, Inc., Hercules, CA), and the pRB bands were colorimetrically visualized.

RESULTS

Tamoxifen and TGF-β1 Effect on Cell Proliferation

We have previously shown that the CAMA-1 cell proliferation was inhibited in a dose-related manner by antiestrogens such as tamoxifen and nafoxidine hydrochloride under various culture conditions [Leung, 1987]. The antiestrogens inhibited cell growth and ^{3}H-thymidine uptake by impeding the progression of cells at the G1 phase

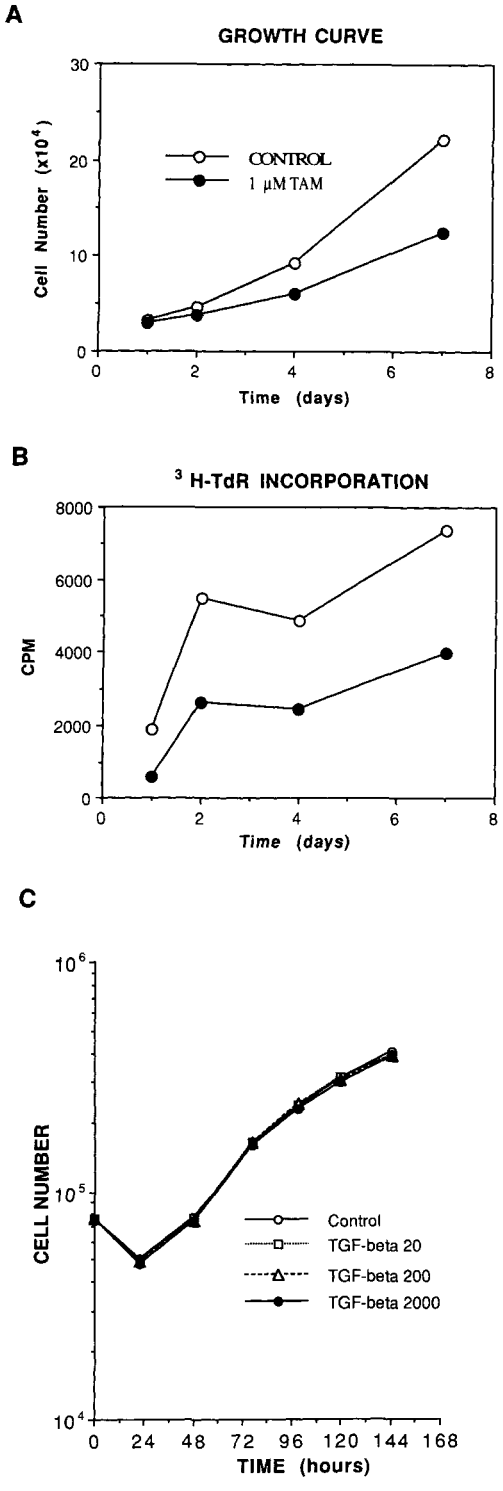

Fig. 1. Effects of tamoxifen and TGF-β1 on CAMA-1 cells. **A:** Growth curves of CAMA-1 cells in the presence or absence of 1 μM tamoxifen added during plating (day 0). Cells were cultured in the presence of 10% serum substitute (Leung, 1987) and 1% dextran-chacoal-treated FBS. **B:** Uptake of ^{3}H-thymidine done in parallel with A. **C.** Growth curves of CAMA-1 cells under various doses of TGF-β1 treatment (ranging from 20 to 2000 pg/ml). Cells were cultured as described in Materials and Methods. All points represent the means of triplicate counts.

resulting in a marked decrease of S-phase and dividing cells per cycle. The inhibitory effect of tamoxifen could be partially reversed by estrogen. To minimize estrogen effect, CAMA-1 cells were grown at time of plating in 10% serum substitute [Leung, 1987] plus 1% dextran-charcoal-treated FBS for evaluating tamoxifen effects on cell proliferation and ^{3}H-thymidine uptake. Even under this suboptimal growth condition, a marked inhibitory effect of tamoxifen (at optimal 1 μM) on both cell number and thymidine uptake was demonstrated (Fig. 1A,B). The possibility of TGF-β modulating the growth inhibitory effect of tamoxifen was investigated at several levels of TGF-β1 that were growth inhibitory in ovarian cancer cell lines such as OVCAR-3 and CAOV-3 [Zhou and Leung, 1992]. TGF-β1, like EGF/TGF-α [Leung et al., 1991], has no effect on cell proliferation of CAMA-1 cells (Figure 1C) even at concentrations as high as 2 ng/ml. However, these concentrations of TGF-β1 were very effective in suppressing cell proliferation of ovarian cancer cell lines [Zhou and Leung, 1992]. The lack of response of CAMA-1 cells to TGF-β differs from that of MCF-7 cells [Knabbe et al., 1987], which re-

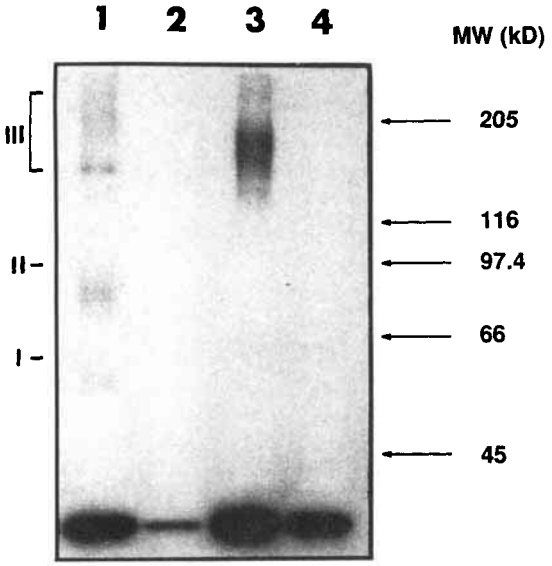

Fig. 2. Determination of TGF-β receptors. Ligand-binding with ^{125}I-TGF-β1 in OVCAR-3 and CAMA-1 cells was analyzed with SDS–PAGE and autoradiographed. Non-specific binding was done with 200-fold excess radioinert ligand. The receptor types are indicated on the left and the free monomeric ^{125}I-TGF-β1 are at the bottom of the gel. The SDS-6H high MW standard mixture was used as the protein marker. *Lane 1,* OVCAR-3: specific ligand-binding; *lane 2,* OVCAR-3: nonspecific ligand-binding; *lane 3,* CAMA-1: specific ligand-binding; *lane 4,* CAMA-1: nonspecific ligand-binding.

spond to growth inhibition of both tamoxifen and TGF-β.

The loss of response of CAMA-1 cells to TGF-β growth inhibition may in part be due to mechanistic defects such as inability to activate the latent form of TGF-β, lack of membrane receptors for the TGF-β, or defects along the intracellular signal transduction pathway [Robert et al., 1988]. These possibilities were then explored.

Determination of TGF-β Receptors

The experiment to determine TGF-β receptors by affinity ligand binding with ^{125}I-TGF-β was conducted according to the method of Massague and co-workers [Massague and Like, 1985; Cheifetz, et al., 1986; Massague, 1987]. The affinity labeling detects three distinct cell-surface binding proteins on most cells [Boyd et al., 1990; Geiser et al., 1992; Kimchi et al., 1988]: the type I (approximately 53 or 65 kD) and the type II (approximately 73 or 95 kD) glycoprotein receptors, and the type III (ranging from 200 to 330 kD) betaglycan receptor. Figure 2 shows that the type I and II receptors were absent and the type III receptor was present in CAMA-1 cells but present in TGF-β-sensitive OVCAR-3 ovarian cancer cells. The expression pattern of TGF-β receptors in CAMA-1 cells is similar to that of DR-mutant and Weri retinoblastoma cells that express type III, but not type I and II receptors [Boyd et al., 1990; Kimchi et al., 1988]. The lack of type I and type II receptors in both DR-mutant and Weri retinoblastoma cells also resulted in these cells' insensitivity to growth inhibition by TGF-β [Boyd et al., 1990].

Detection of TGF-β Type II Receptor mRNA

The absence of TGF-β type I and II receptors in CAMA-1 cells might be attributed to the lack of transcription or translation of the corresponding receptor genes or to defects in the process of posttranslational modifications. Recent cloning and sequencing of human TGF-β type II receptor cDNA [Lin et al., 1992] has made it feasible to examine the presence of the type II receptor mRNA by the ID RNA/PCR procedure [Ji et al., 1990]. A cDNA segment of 120 bp located at the N-terminal region of the receptor was chosen for amplification. As shown in Figure 3A, the TGF-β type II receptor mRNA transcript was absent in CAMA-1 cells regardless of whether or not estrogen or tamoxifen was present in the culture. Concurrent analysis of c-myc mRNA transcript of these CAMA-1 cells (Fig. 3B) was positive, indicative of procedural integrity. Furthermore, positive controls from plasmid H2-3FF and other known TGF-β responsive cell lines (MCF-7_{MI}, OVCAR-3, and CAOV-3), showed clear PCR products of type II receptor at 120 bp (Fig. 3A). The extreme sensitivity of the ID RNA/PCR method precludes the possibility that low levels of type II receptor mRNA might be present in

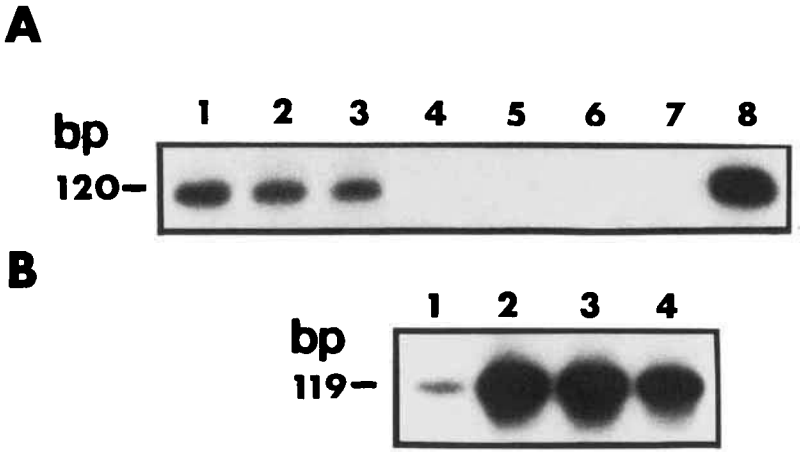

Fig. 3. Intron-differential RNA/PCR analysis of TGF-β type II receptor gene expression. **A:** Southern analysis of the 120 bp ID RNA/PCR products. *Lane 1,* CAOV-3; *lane 2,* OVCAR-3; *lane 3,* MCF-7_{MI}; *lane 4,* CAMA-1: cultured in DMEM/F12 medium containing phenol-red, 15% FBS, and 1 nM 17β-estradiol; *lanes 5–7,* CAMA-1: cultured in phenol-red-free DMEM/F12 containing 15% dextran-charcoal stripped FBS with 5. vehicle control (95% ethanol); *lane 6,* estrogen-treated (10 nM 17β-estradiol); *lane 7,* tamoxifen-treated (1 μM tamoxifen); *lane 8,* positive control of PCR amplification from H2-3FF plasmid which containing the human TGF-β type II receptor cDNA clone. **B:** Concurrent ID RNA/PCR analysis of c-*myc* oncogene expression of CAMA-1 cells. Samples analyzed in lanes 1–4 correspond to those of lanes 4–7 in A, except the c-*myc* primers and probe [Ji et al., 1990] were used. The blots were exposed for 2–2.5 h. Longer exposures of blot A did not reveal any 120-bp Southern bands in CAMA-1 cells (lanes 4–7).

some CAMA-1 cells. Nevertheless, the lack of detectable type II receptor gene transcript could be accounted for by rapid degradation of the mRNA, improper processing, or the cells' inability to transport the transcript out of the nucleus. Our results demonstrate that TGF-β type II receptor gene transcript was not detected in CAMA-1 cells whether they were treated with estradiol or tamoxifen.

Southern Analysis of TGF-β Type II Receptor Gene

The lack of TGF-β type II receptor mRNA could arise from a number of factors such as lack of transcription or splicing processing, or rapid degradation of the mRNA. Transcription of TGF-β type II receptor gene would not take place in the absence, deletion or rearrangement of this gene or with defects in the machinery for transcribing this gene. To investigate possible defects in the type II receptor gene structure, we performed Southern analysis on the CAMA-1 cells, and compared the results to those of Southern analysis on MCF-7_{MI} and OVCAR-3 cells which contain the normal type II receptor.

The genomic DNA of CAMA-1, MCF-7_{MI} and OVCAR-3 cells were digested with excess endonucleases *Bam*HI, *Eco*RI, and *Hin*dIII and resolved on a 1% agarose gel. The fractionated DNAs were blotted onto the GeneScreen Plus nylon membrane and probed with human TGF-β type II receptor probe (4.5 kb *Eco*R1 fragment from H2-3FF plasmid). Figure 4 shows that regardless of which endonuclease was used, the CAMA-1 cells have Southern bands similar to those of MCF-7_{MI} and OVCAR-3, both of which express the normal TGF-β type II receptor. Moreover, the intensities of the Southern bands of CAMA-1 were comparable to those of MCF-7_{MI} and OVCAR-3 cells, indicative of the same copy number of this gene in each of these cell lines. The Southern result suggests that the CAMA-1 cells possess an apparently normal TGF-β type II receptor gene, although subtle structural abnormality cannot be excluded.

Analysis of Retinoblastoma Gene and Products

The fact that the CAMA-1 cells do not express the TGF-β type II receptor despite having an apparently normal gene suggests that the lack of this gene expression may be due to reasons other than gene deletion or absence. A previous report by Kimchi et al. [Kimchi et al., 1988] has implicated an intimate relationship between the expression of TGF-β receptors and that of the

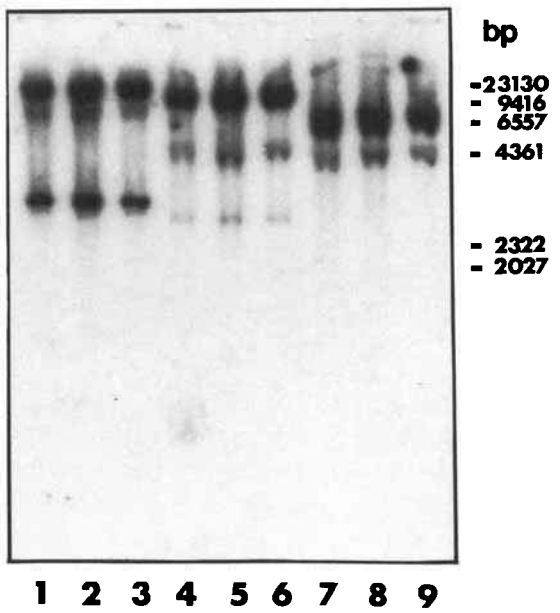

Fig. 4. Southern analysis of TGF-β type II receptor gene. *Lanes 1–3,* the genomic DNAs were digested with excess endonuclease *Bam*HI; *lanes 4–6, Eco*RI; *lanes 7–9, Hin*dIII. The DNA digests from OVCAR-3 (*lanes 1,4,7*), MCF-7_{MI} (*lanes 2,5,8*), and CAMA-1 (lanes 3, 6, and 9) were loaded to each lane, respectively, and probed with the 4.5 kb human TGF-β type II receptor gene probe from H2-3FF. The λDNA/*Hin*dIII digest was used as DNA marker. The blot was a 20-h exposure.

retinoblastoma (Rb-1) gene: the TGF-β receptors and Rb gene products are both absent in the retinoblastoma cells but are both present in normal retinal cells. It is plausible that the lack of TGF-β receptor expression may be related, directly or indirectly, to the absence of normal Rb gene or its products. This possibility has been explored by Southern and Western blotting analyses of Rb gene and its products in the CAMA-1 cells, respectively. The genomic DNAs of CAMA-1 cells and other control cells were digested with excess endonuclease *Hin*dIII. By Southern analysis, using either 0.9-kb or 3.8-kb pRb probes, the presence of a normal Rb gene in the CAMA-1 cells was demonstrated (Fig. 5A,B). Results from Western analysis were consistent with the expression in CAMA-1 cells of normal pRB proteins, both hyper-phosphorylated and hypo-phosphorylated species (Fig. 6). These results rule out the possible contribution of an abnormal Rb gene and its products in the turning-off of TGF-β type II receptor gene transcription in CAMA-1 cells.

DISCUSSION

Antiestrogens such as tamoxifen are choice adjuvant therapeutic agents in the treatment of

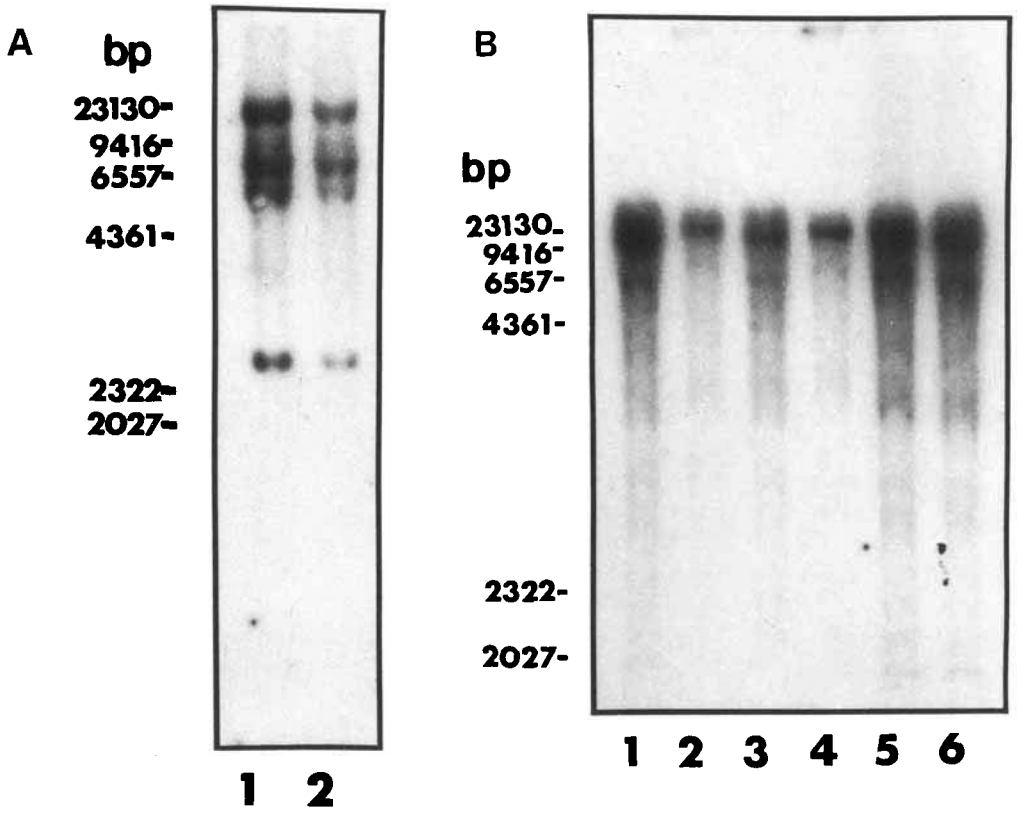

Fig. 5. Southern analysis of retinoblastoma gene. The genomic DNAs were digested with excess Hind III endonuclease and probed with (A) 3.8-kb and (B) 0.9-kb Rb probes, respectively. A: *lane 1,* CAMA-1; *lane 2,* MCF-7_{MI}. B: *Lane 1,* MCF-7_{MI}; *lane 2,* MCF-7_{NIH}; *lane 3,* CAMA-1; *lane 4,* Hs578T; *lane 5,* CAOV-3; *lane 6,* OVCAR-3. The A blot was exposed for 2 days and the B blot for 3 days.

estrogen-responsive breast cancers [Lerner and Jordan, 1990; Hamm and Allegra, 1991]. That tamoxifen has a tumoristatic action rather than a tumoricidal effect on the tumor growth has been documented in many studies. The underlying mechanism by which antiestrogens exert tumoristatic inhibition on breast tumor growth is still unknown. Results from early studies pointed to a possible relationship between antiestrogen action and TGF-β-related events but subsequent investigation have yielded conflicting results. Antiestrogens are found to induce the secretion of auto-inhibitory TGF-β in MCF-7 cells at the posttranscriptional level [Knabbe et al., 1987; Arrick et al., 1990], making TGF-β expression a good marker for antiestrogen action [Knabbe et al., 1991]. In vivo, tamoxifen induces localized secretion of TGF-β between and around stromal fibroblasts [Butta et al., 1992], and TGF-β in turn can inhibit terminal end bud growth and development of the mouse mammary gland (Salomon et al., 1989). Elevated TGF-β production is generally associated with enhanced tumor suppressing activities. However, the modulation of TGF-β production in oncogene-transformed human mammary epithelial cells does not correlate with the extent of malignant transformation [Valverius et al., 1989], and the level of TGF-β mRNA expression in tumors does not necessarily correspond to patients' responsiveness to tamoxifen therapy [Thompson et al., 1991]. Furthermore, several studies have suggested that TGF-β may have a net effect in favoring the maintenance, progression, or metastasis of mammary tumor cells in vivo [see review by Arteaga and Coffey, 1992]. Collectively, TGF-β may contribute to the overall action of tamoxifen in cells which make and respond to TGF-β, however, there is no conclusive evidence that TGF-β is required for tamoxifen action in estrogen-targeted cells.

The effect of TGF-β on human breast cancer cell proliferation has been studied in a number of breast cancer cell lines. Estrogen-nonresponsive and estrogen receptor (ER)-negative breast cancer cell lines MDA-MB-231, MDA-MB-330, MDA-MB-468, SKBR-3, Hs578T, and BT-20 are inhibited by TGF-β [Arteaga et al., 1988b; see

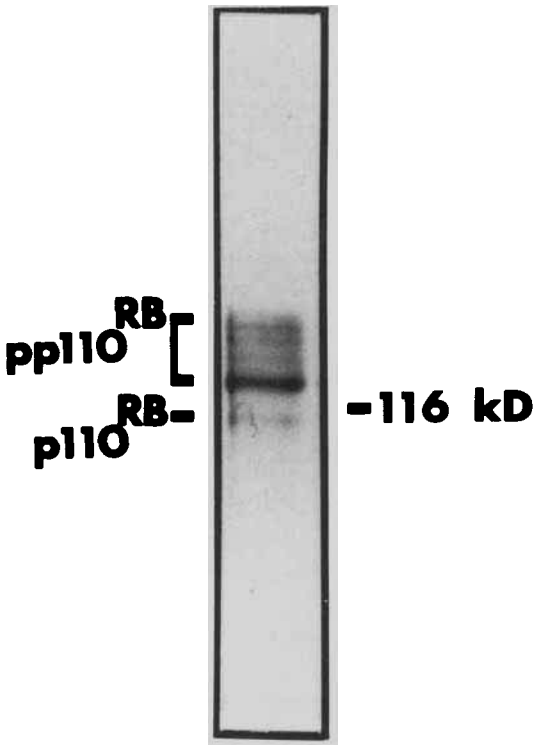

Fig. 6. Western analysis of retinoblastoma gene products in CAMA-1 cells. pRB proteins in CAMA-1 cells were analyzed by immunoprecipitation and immunoblotting with anti-RB monoclonal antibody (Ab-4) from Oncogene Science. Both hyper- and hypo-phosphorylated pRB protein species were detected on the blot. The SDS-7B prestained SDS standard mixture was used as a protein MW marker.

also reviews by Zugmaier and Lippman, 1990 and by Salomon et al., 1989]. For estrogen-responsive and ER-positive breast cancer cell lines, TGF-β effect remains controversial [Zugmaier and Lippman, 1990]. While anchorage-independent growth of MCF-7 cells was inhibited by TGF-β [Manni et al., 1991], several studies have shown that TGF-β is ineffective in inhibiting ER-positive cell lines [Arteaga et al., 1988b; Murphy and Dotzlaw, 1989; Cohen et al., 1990; see also a review by Zugmaier and Lippman, 1990], and in all these cases the TGF-β receptors are also absent in the cells (Table I). Our results are in agreement with those of Osborne's group [Arteaga et al., 1988b] in that the CAMA-1, an estrogen-responsive, ER-positive, and antiestrogen-sensitive breast cancer cell line, is not inhibited by TGF-β, presumably due to a lack of TGF-β type I and II receptors. Our results indicate that the TGF-β-regulated events are not a mandatory requirement for antiestrogen action in inhibiting cell proliferation and TGF-β does not function as an autocrine growth-inhibitory factor in mediating antiestrogen action in CAMA-1 cells. The fact that the CAMA-1 cells do express the TGF-β1 gene [Ji et al., 1990] suggests that this factor might act originally in the patient as a paracrine factor in modulating cellular events of adjacent cells to the cancer.

Both type I and II receptors of TGF-β have been demonstrated to be essential for TGF-β modulation of cell proliferation [Boyd and Massague, 1989; Geiser et al., 1992]. Our findings of CAMA-1 insensitivity to TGF-β and the absence of type I and II receptors are in agreement with results of these investigators. Recent cloning of the human type II receptor revealed that this receptor is a functional transmembrane serine/threonine kinase [Lin et al., 1992]. The lack of type II receptor mRNA, and thus the type II receptor protein, precludes any inhibitory signal of TGF-β from being transduced into the cells to elicit an inhibitory growth response. Southern analysis revealed that the CAMA-1 cells have a normal TGF-β type II receptor gene and the lack of its mRNA is not due to absence or deletion of this gene. Our study demonstrated for the first time that the absence of type II receptor protein is due to the lack of mRNA, presumably inhibited at the pre-translational level. By the extremely sensitive ID RNA/PCR method, we have shown that TGF-β type II receptor mRNA was absent in these cells with or without estrogen or antiestrogen treatment. The mechanism by which type II receptor mRNA is absent in CAMA-1 cells requires further investigation.

Recent advent of study on the products of tumor suppressor genes has stimulated investigation on the relationship between TGF-β action and that of the retinoblastoma (Rb-1) gene [Moses et al., 1990; Roberts et al., 1991]. The linkage of TGF-β inhibitory action and Rb gene function was first suggested in the report by Kimchi et al. [1988], which stated that the TGF-β receptors were absent in the retinoblastoma cells but present in normal retinal cells. The TGF-β was demonstrated to control pRB function by blocking pRB phosphorylation [Laiho et al., 1990]. In turn, the pRB was able to modulate TGF-β action by regulating the expression of TGF-β through a retinoblastoma control element (RCE) [see review by Roberts et al., 1991]. The ability of TGF-β to down-regulate c-*myc* expression in keratinocyte cells was abrogated by viral transforming proteins that formed complexes with the pRB [Pietenpol et al., 1990].

TABLE I. Interrelationship Between Responsiveness to Tamoxifen and TGF-β and Presence of TGF-β Receptors (TGFβR) and Rb Gene Products (pRB)

Response to				
TAM	TGF-β	TGFβR/pRB	Examples	References
Yes	Yes	$TGF\beta R^+/pRB^+$	MCF-7[a]	Knabbe et al., 1987
Yes	No	$TGF\beta R^-/pRB^+$	CAMA-1 MCF-7[a] etc.	Ji et al., this report Arteaga et al., 1988b
No	Yes	$TGF\beta R^+/pRB^+$	Hs578T, etc. LY2?[b]	Arteaga et al., 1988b Lippman et al., 1987 T'Ang et al., 1988
No	Yes	$TGF\beta R^+/pRB^-$	MDA-MB-436 MDA-MB-468	Horowitz et al., 1990 Ong et al., 1991
?	?	$TGF\beta R^-/pRB^-$	?	Not reported

[a]All MCF-7 sublines tested have a normal Rb gene and its products [Horowitz et al., 1990, T'Ang et al., 1988]; however, the TGF-β receptors are present in some of the sublines and absent in others [Arteaga et al., 1988b, Boyd et al., 1990, Knabbe et al., 1987].
[b]LY2 is an antiestrogen-resistant and TGF-β-sensitive MCF-7 subline.

However, it is not known whether the TGF-β receptors are regulated by pRB. In contrast to these results, there is evidence that TGF-β and pRB growth suppressive actions are not directly related. For example, two breast cancer cell lines, MDA-MB-436 and MDA-MB-468, which lack functional pRB proteins due to gene deletion or rearrangement of Rb [Lee et al., 1988; T'Ang et al., 1988], contain TGF-β receptors and are growth-inhibited by TGF-β [Ong et al., 1991]. These findings showed that the pRB action is not an obligatory event in the pathway of growth inhibition by TGF-β. Cancer cells which do not contain functional Rb products are fully capable of responding to TGF-β inhibition.

Concurrent examination of TGF-β receptor and Rb gene expression in different model systems has been conducted. This interrelationship can be summarized as follows: in most cells examined, TGF-β receptors and pRB proteins are both present; in retinoblastoma cells, TGF-β receptors and Rb gene products are both absent; and in two breast cancer cell lines (MDA-MB-436 and MDA-MB-468), only the TGF-β receptors are present while Rb gene products are absent due to deletion or rearrangement. The CAMA-1 cells present yet another scenario wherein normal pRB proteins and Rb gene are present while the TGF-β type I and II receptors are absent. Our results are in agreement with the finding of Gullick's group [Ong et al., 1991] that the relationship between TGF-β system and Rb function is indirect due to the nonconcurrent presence or absence of TGF-β and Rb actions. Although there is strong evidence of the interregulation of the TGF-β and Rb gene systems, the findings of Gullick et al. suggest that TGF-β inhibition of cell proliferation is not likely mediated by the action of pRB proteins. What cell proliferation-related events are common to the actions of these two growth suppressive systems is unknown.

The relationship between tamoxifen inhibition and tumor suppressive effect of Rb products in human breast cancer cells have not been investigated. This study is the first to simultaneously examine the presence of TGF-β receptors and Rb gene products in view of Rb's possible relationship to tamoxifen and TGF-β responsiveness. The presence of these molecules, examined in separate studies, in relation to breast cancer cells' responsiveness to TGF-β and tamoxifen is summarized in Table 1. Although the presence of pRB is not essential to TGF-β responsiveness, as demonstrated by pRB-negative, TGF-β b-sensitive MDA-MB-436 and MDA-MB-468 cell lines, the requirement of pRB for tamoxifen inhibition of cell proliferation is currently unknown. The facts that the pRB is present in all tamoxifen-inhibited breast cancer cell lines such as MCF-7 and CAMA-1 (Table I), and that the pRB appears normally phosphorylated in CAMA-1 cells (Fig. 6) suggest that pRB might be involved directly or indirectly in tamoxifen-mediated growth arrest. This assumption, however, requires further investigation.

It is interesting to note that the estrogen- and antiestrogen-responsive CAMA-1 cells are insen-

sitive not only to EGF/TGF-α stimulation but also to TGF-β inhibition. The membrane receptors for EGF/TGF-α and TGF-β are both missing in the CAMA-1 cells. These results suggest that the transforming growth factors (TGFs) are not autocrine mediators for estrogen and antiestrogen actions and thus TGFs are not obligatory components in these hormones' regulation of CAMA-1 breast cancer cells. Furthermore, this unique feature of CAMA-1 cells presents a model system to investigate the molecular events of estrogen and tamoxifen action without the interference of the TGFs. We have postulated previously [Leung et al., 1991] that convergent points might exist along the pathways of estrogen and TGF-α action in promoting tumor growth in other cell systems; similar convergent points may also exist along the pathways of anti-estrogen and TGF-β action in suppressing tumor growth. Identification of these convergent points may provide further insight regarding the interactions of these growth modulators.

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