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# Tamoxifen and Estrogen Effects on TGF-$\beta$ Formation: Role of Thrombospondin-1, $\alpha v\beta 3$, and Integrin-Associated Protein

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**We have found that the enhanced activation of latent TGF-$\beta$ by human breast carcinoma cell lines either treated with tamoxifen or deprived of estrogen is dependent upon thrombospondin-1 (TSP-1) since activation was blocked by anti-TSP-1 antibodies or by a TSP antagonist peptide. However, TGF-$\beta$ formation upon tamoxifen exposure to estrogen withdrawal is associated with decreased levels of soluble TSP-1. A concomitant increase in the expression of the TSP-1 receptors $\alpha v\beta 3$ and integrin-associated protein (IAP) occurs under these conditions, and antibodies to TSP-1 or to these receptors inhibit increased TGF-$\beta$ formation. Therefore, increased cell surface associated TSP-1 enhances latent TGF-$\beta$ activation.** 

***Key Words:*** **TGF-$\beta$; tamoxifen; estrogen; thrombospondin; $\alpha v\beta 3$; integrin-associated protein.**

The TGF-$\beta$s have multiple activities ranging from inhibition of growth, suppression of the immune system, inhibition of inflammation, and stimulation of matrix biosynthesis (18, 24). Although TGF-$\beta$1, $\beta$2 and $\beta$3 are widely expressed, the TGF-$\beta$s are usually found in a latent form (25) due to the continued association of the cleaved TGF-$\beta$ propeptide with the growth factor by noncovalent forces even after secretion. In this complex TGF-$\beta$ cannot interact with its receptors.

A number of mechanisms have been proposed for the activation of latent TGF-$\beta$. These include proteolytic degradation of the TGF-$\beta$ propeptide (21, 22, 33, 38), association of the propeptide with the integrin $\alpha v\beta 6$ (26), ionizing radiation or free radicals (5, 6, 12), high or low pH (8, 22), and interaction with TSP-1 (27, 30, 34–36). The molecular characteristics of these activation mechanisms are not understood, even though there is *in vivo* evidence pointing to the significance of certain pathways (9, 26).

Here we report that treatment of the human breast cancer cell line T47-D either by exposure to the antiestrogen tamoxifen or by the removal of estrogen from the culture medium results in a 2- to 3-fold increase in active TGF-$\beta$. This enhanced activation of latent TGF-$\beta$ requires TSP-1 as well as the associated TSP-1 receptors $\alpha v\beta 3$ and IAP. This is the first demonstration of nonproteolytic TSP-1-dependent activation of latent TGF-$\beta$ that requires select TSP-1 receptors.

## METHODS

*Cell culture.* T47-D cells (ATCC, Rockville, MD) were maintained in DMEM + 5% FCS. Prior to experiments, cells were cultured for 5 days in DMEM with either 5% FCS or 5% dextran-coated charcoal-treated FCS (14) to deplete cells of estrogen. 100,000 cells/cm$^2$ were plated in the indicated serum, allowed to attach for 24 h, washed with PBS, and incubated for 24 h in DMEM–0.1% BSA to remove remaining serum components. Cells were then incubated under the experimental conditions with either 1 mM tamoxifen or 10 nM $\beta$-estradiol for 48 h at 37° in 5% $CO_2$. For the agar growth studies, T47D human breast adenocarcinoma cells (ATCC) were maintained in RPMI with 0.2 I.U./mL insulin and 10% FBS (Hyclone, Logan, UT). NRK-49F cells (ATCC) were cultured (passages 18 to 24) in DMEM with 4.5 g/L glucose plus 10% CS (Hyclone). All studies were done in triplicate. Error bars indicate the standard deviation.

*Assays for TGF-$\beta$.* TGF-$\beta$ was quantified using a plasminogen activator inhibitor-1 (PAI-1)–luciferase-based assay (2). Assay results were expressed in pg/mL or were normalized to the TGF-$\beta$ activity of control cells. Specificity was ascertained by the addition of 10 $\mu$g/mL pan-neutralizing TGF-$\beta$ monoclonal antibody (Celtrix). Additional assays for TGF-$\beta$ were performed using the NRK growth assay (28).

*Metabolic labeling and immunoprecipitation of conditioned medium for TSP-1.* T47-D cells were incubated in methionine/cysteine-free medium containing 125 $\mu$Ci/mL [$^{35}$S]methionine/

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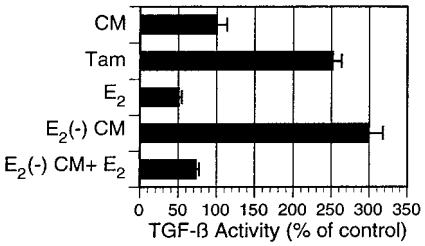


**FIG. 1.** Effect of estrogen deprivation or tamoxifen addition on TGF-$\beta$ activity in T47-D cultures. T47-D cells were cultured and the conditioned medium (CM) was assayed for TGF-$\beta$. TGF-$\beta$ was quantified using a standard curve developed with recombinant human TGF-$\beta$1. The activities obtained were normalized using CM from cells grown in FCS as 100%. The 100% value was equivalent to 8.3 pg/mL.

cysteine Express label (New England Life Sciences, Boston, MA), 2.5% dialyzed FCS, and 1% regular medium for 16 h. After collection and clarification by centrifugation, 50 $\mu$g/mL aprotinin, 100 $\mu$g/mL PMSF, and 10 $\mu$g/mL pepstatin A were added. Samples were normalized by TCA precipitable counts prior to immunoprecipitation (15) with 1.0 $\mu$g/mL anti-TSP-1 antibody (45.2, R. Silverstein, Weill–Cornell University Medical College, New York, NY). Samples were rotated overnight at 4°C, and incubated with Protein G-agarose for 1 h at 4°C. After centrifugation, the protein in the pellets was recovered, analyzed by SDS–PAGE and revealed by autoradiography.

*Enzyme-linked immunosorbent assay for TSP-1.* TSP-1 was assayed using an ELISA (10).

*RNA isolation and Northern blotting.* Total RNA was harvested from T-47-D cells using TriPure (Boehringer Mannheim). The RNA was quantified by OD at 260 nM. Total RNA (15 $\mu$g/lane) was subjected to 1% agarose/formaldehyde gel electrophoresis (4), transferred and crosslinked to nylon membranes (Boehringer, Mannheim). Radiolabeled DNA probes for TGF-$\beta$1, $\beta$2, $\beta$3 or TSP-1 were prepared using Ready-to-Go Reactibeads (Pharmacia). The membranes were subjected to Northern analysis using pre-hybridization/hybridization buffers (5 Prime → 3 Prime Inc., Boulder, CO). After low and high stringency washes, the membranes were exposed to XAR film for 2 days at −80°C with intensifying screens.

*Fluorescence-activated cell sorting.* Cells cultured under the indicated conditions were detached (PBS plus 5 mM EDTA), diluted, and incubated with the indicated monoclonal antibodies for 30 min at 4°C. Cells were washed, resuspended in 5% estrogen-depleted FCS containing goat anti-mouse antibody conjugated to R-phycoerythrin (1:15; Dako Corp., Carpinteria, CA) for 30 min, washed in 1% paraformaldehyde in PBS, and analyzed using a FACScan analyzer (Becton–Dickinson).

## RESULTS AND DISCUSSION

To characterize the effects of tamoxifen addition or estrogen depletion on latent TGF-$\beta$ activation, T47-D human breast cancer cells were cultured as described. In the presence of tamoxifen, there was a 2.5-fold increase in TGF-$\beta$ activity as compared to the control (Fig. 1), as reported earlier (19). When cultured in estrogen-depleted serum, there was a 3-fold increase in active TGF-$\beta$ and a 4-fold increase compared to levels found in cells supplemented with estrogen. Although both tamoxifen addition and estrogen depletion increase the total amount of TGF-$\beta$ produced, the latent TGF-$\beta$ in the active form increased by 1.6- and 2-fold, respectively. Using TGF-$\beta$ isoform specific antibodies as well as antibodies to the latent TGF-$\beta$ binding protein, we found that the predominant form of TGF-$\beta$ produced was the latent complex of TGF-$\beta$3 plus LTBP-1 (data not shown).

To establish the mechanism of latent TGF-$\beta$ activation we tested the ability of inhibitors to block tamoxifen-induced latent TGF-$\beta$ activation. Inhibitors against serine, cysteine and metallo proteases had no effect nor did excess mannose 6-phosphate, antibodies to LTBP-1, or transglutaminase inhibitors (data not shown); all agents that block TGF-$\beta$ formation (11, 13, 29, 32). These treatments also did not inhibit the activation of latent TGF-$\beta$ in estrogen-depleted cultures.

TSP-1, a soluble and extracellular matrix glycoprotein, can activate latent TGF-$\beta$ (27, 28, 34–37). We, therefore, measured the effect of anti-TSP-1 antibodies. The monoclonal antibody 133 (mAb 133) reduced TGF-$\beta$ activity by 50% (Fig. 2A). This is a 70% inhibition of the increase in TGF-$\beta$ activity observed after estrogen deprivation. A second monoclonal antibody against the N-terminal heparin-binding domain of TSP-1 (mAb 2.5) and a polyclonal rabbit anti-TSP-1 antibody (R. Silverstein) also reduced TGF-$\beta$ activity by 50 and 40%, respectively (data not shown). A control

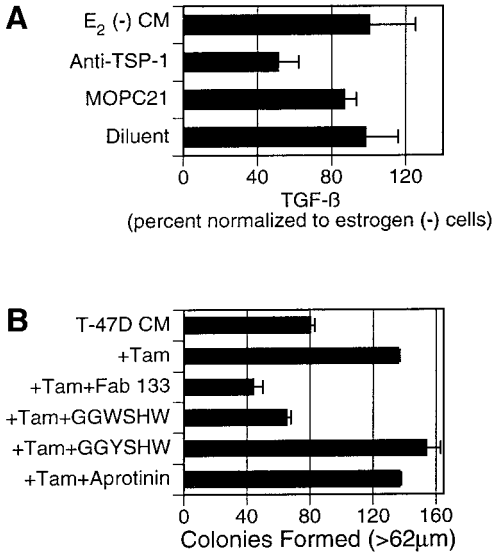


**FIG. 2.** TSP-1 antagonists inhibit latent TGF-$\beta$ activation. (A) T47-D cells were cultured in estrogen-depleted serum. Monoclonal anti-TSP-1 (mAb 133), MOPC21, an isotype control, or diluent was added at a final concentration of 25 $\mu$g/mL for the final 48 h of incubation. The CM was assayed for TGF-$\beta$ activity, and the results were normalized to estrogen-depleted CM. (B) T47-D cells were cultured for 24 h in the presence or absence of 10 nM tamoxifen. mAb 133 Fab (10 $\mu$g/ml), GGWSHW (14 $\mu$M), GGYSHW (31 $\mu$M), or aprotinin was added to cultures for this 24-h period. These reagents did not affect TGF-$\beta$ activity under basal conditions. Aliquots of CM were tested for TGF-$\beta$ activity in the NRK assay. Baseline colony formation in the presence of 1 nM EGF was 46 colonies.

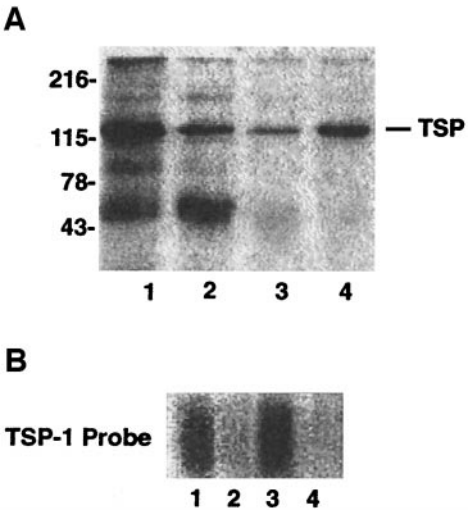


**FIG. 3.** Estrogen deprivation decreases TSP-1 levels in conditioned medium of T47-D cells. (A) SDS–PAGE of immunoprecipitates of metabolically labeled TSP-1 from estrogen-depleted or tamoxifen-treated T47-D cultures. T47-D cells were grown and metabolically labeled, and equivalent amounts of radioactivity were immunoprecipitated with monoclonal anti-TSP-1 antibody (mAb 45.2). Equivalent amounts of radioactivity were added to each well. Lane 1, T47-D CM; Lane 2, tamoxifen-treated T47-D CM; Lane 3, estrogen-depleted T47-D CM; Lane 4, CM from estrogen-depleted T47-D cells incubated for the final 48 h with 50 nM $\beta$-estradiol. MW standards are indicated on the left; TSP-1 location is indicated on the right. (B) Northern analysis of tamoxifen-treated and estrogen-depleted T47-D cells using a TSP-1 probe. Tamoxifen-treated or estrogen-depleted T47-D cells were cultured as described. $\beta$-Estradiol (50 nM) was added to one sample for the final 48 h of incubation. Lane 1, T47-D control RNA; Lane 2, tamoxifen-treated T47-D RNA; Lane 3, $\beta$-estradiol-treated T47-D RNA; Lane 4, estrogen-depleted T47-D RNA.

antibody, MOPC21 (Sigma, St. Louis, MO), had no significant effect. In another series of experiments, using different culture conditions (10 nM tamoxifen, 24 h treatment), either mAb 133 or a peptide from the TSP type 1 repeats that blocks activation of TGF-$\beta$ by TSP-1, reduced TGF-$\beta$ to basal levels (Fig. 2B). The inactive peptide (GGYSHW) or aprotinin had no effect on TGF-$\beta$ levels. The additions by themselves had no effect on colony formation. These results indicate that estrogen depletion and tamoxifen treatment stimulate TGF-$\beta$ formation via a TSP-mediated mechanism consistent with findings of TSP-1-dependent activation of latent TGF-$\beta$ in tamoxifen-treated MCF-7 cells (27).

When the medium from estrogen-depleted cultures was analyzed for TSP-1 using either immunoprecipitation and SDS-PAGE (Fig. 3A) or by ELISA (data not shown), a significant decrease in TSP-1 levels was observed. Northern blotting of mRNA extracted from tamoxifen-treated or estrogen-deprived T47-D cells also revealed a 10-fold decrease in TSP-1-mRNA (Fig. 3B). Therefore, activation of latent TGF-$\beta$ occurred under conditions of decreasing TSP-1 levels. Similar findings were observed with tamoxifen-treated MCF-7 cells (data not shown).

We reasoned that TGF-$\beta$ formation might result from increased cell association of TSP-1. Therefore, we assessed the ability of antibodies against different TSP-1 receptors or receptor binding domains on TSP-1 to affect latent TGF-$\beta$ activation. Antibodies against TSP-1 (mAb 133), against the carboxy-terminal cell binding domain (CBD) of TSP-1 (mAb C6.7; W. Frazier, Washington University Medical School, St. Louis, MO), and against the TSP-1 receptors $\alpha v\beta 3$ (mAb 7E3; B. Coller, Mt. Sinai Medical School, NY), the integrin-associated protein (mAb B6H12; E. Brown, Washington University Medical School), and CD36 (J. Barnwell, NYU School of Medicine, NY) were added to estrogen-depleted cultures. The medium was removed after 48 h and assayed for TGF-$\beta$. The results (Fig. 4) indicate that anti-TSP-1, anti-CBD, anti-IAP, and anti-$\alpha v\beta 3$ antibodies decreased TGF-$\beta$ formation, whereas anti-CD36 and the irrelevant antibody, MOPC21, had no significant effect, indicating that TSP-1 and specific TSP-1 receptors are involved in the activation of latent TGF-$\beta$. There was no significant change in total TGF-$\beta$ produced under these conditions. To confirm the presence of $\alpha v\beta 3$, IAP, and CD36 on the cell surface, FACS analysis of control and estrogen-depleted T47-D cells was performed using receptor antibodies. The estrogen-depleted cells showed a 2-fold increase in cell surface-associated TSP-1, $\alpha v\beta 3$, and IAP compared to controls (data not shown). There was no significant change in the expression of CD36. Similar results were found with tamoxifen-treated cells (data not shown).

TSP-1 plays an important role in TGF-$\beta$ formation, but other cellular factors can modulate the ability of TSP-1 to activate latent TGF-$\beta$. Thus, TGF-$\beta$ formation by bleomycin-stimulated alveolar macrophages requires plasmin and TSP-1 binding to the CD36 receptor (40). Other studies with thrombin-stimulated

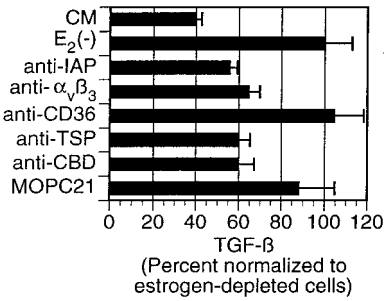


**FIG. 4.** Antibodies against TSP-1 and its receptors inhibit activation of latent TGF-$\beta$. T47-D breast cancer cells were cultured as described. During the final 48 h of incubation, monoclonal antibodies directed against TSP-1 (mAb 133), the CBD domain of TSP-1 (mAb C6.7), the TSP-1-binding integrin $\alpha v\beta 3$ (mAb 7E3), the $\alpha v\beta 3$-associated IAP (mAb B6H12), CD36, and MOPC21 were added at 20 $\mu$g/mL to the estrogen-depleted cultures. The CM was assayed for TGF-$\beta$ by the PAI-1 luciferase assay. Results are normalized to the estrogen-depleted CM. $E_2(-)$: estrogen-depleted conditioned medium. The 100% value was equivalent to 27.4 pg/mL.

platelets from wild type and TSP-1 knockout mice demonstrate no significant difference in active TGF-$\beta$ even though TSP-1 and latent TGF-$\beta$ are stored in the same platelet granules (40). These systems indicate that some pools of TSP-1 may not be in a conformation permissive for activation of latent TGF-$\beta$. Binding of TSP-1 to cell surface receptors, post-translational modifications (17), and/or possible interactions with proteins which alter the TGF-$\beta$ binding sites in TSP-1, may alter TSP-1 conformation allowing activation of latent TGF-$\beta$. These steps would introduce an additional measure of control on the activation of this potent growth regulator.

The development of tamoxifen-resistant cancers is a major problem in the treatment of this disease. Evidence suggests that increases in TGF-$\beta$ activity are directly related to tamoxifen resistance (3, 16, 39). TGF-$\beta$-dependent down-regulation of NK cell activity contributes to progression of tamoxifen-resistant tumors (3, 20) and is implicated in radiation-induced fibrosis (7). Identification of the mechanism controlling TGF-$\beta$ bioactivation is thus critically important. Antagonists of TSP-1-dependent latent TGF-$\beta$ activation represent a novel strategy for altering tamoxifen resistance.

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