Advances in Brief

Tumor Necrosis Factor α Enhances Secretion of Transforming Growth Factor β2 in MCF-7 Breast Cancer Cells

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Abstract

We studied the effect of tumor necrosis factor α (TNF-α) on transforming growth factor β (TGF-β) secretion by human breast cell lines to further characterize the antitumor effects of TNF-α. We found that TNF-α increased the secretion of TGF-β in two established breast cancer cell lines (MCF-7 and ZR-75-1) but not in two immortalized human mammary epithelial cell lines (184B5 and MCF-10A). In MCF-7 cells, TNF-α increased the secretion of total TGF-β 6.1-fold within 72 h in a dose-dependent manner. The secretion of both latent and active forms of TGF-β was increased, and their ratio altered from 25:1 to 12:1 in the medium. TNF-α converted the secretory pattern of TGF-β by MCF-7 cells from the heterodimeric form TGF-β1,2 to the homodimeric form TGF-β2.

Immunoblot analysis under nonreducing conditions identified four molecular mass species of TGF-β secreted in the culture media of untreated MCF-7 cells (238, 210, 40-55, and 25 kDa). Under reducing conditions, three molecular mass species of TGF-β were identified: 88, 44, and 12 kDa. Gel filtration analysis demonstrated that the secreted TGF-β within the range of 12-88 kDa was biologically active. TNF-α treatment did not alter the size of molecular mass species secreted by MCF-7 cells and did not change steady-state levels of mRNA for TGF-β1 or TGF-β2. These findings indicate that TNF-α may regulate quantitatively and qualitatively TGF-β secretion by human breast cancer cells in vitro. The diverse biological activities of TGF-β may also allow TNF-α to regulate the growth and metabolism of human mammary epithelial cells and/or stromal cells in a paracrine manner.

Introduction

TNF-α² is an important modulator of the growth and metabolism of many tumor types in vitro and in vivo. TNF-α causes necrosis of methylcholanthrene-induced sarcomas (1), B16BL6 melanoma (2), ovarian carcinoma xenografts (3), and human breast cancer explants (4) in mice. Tumor inhibition may represent a direct effect of TNF-α on tumor, or it may represent indirect actions on the endothelium or immune system (2, 5). Importantly, TNF-α may also regulate cytokine or growth factor secretion by tumor cells, controlling cell growth in an autocrine or paracrine manner (2). Among the most responsive tumor cells to TNF-α in vitro are human breast cancer cells (6). We have shown that TNF-α inhibits the growth of MCF-7 cells in vitro, blocks cell cycle progression at G0/G1, prevents estradiol-stimulated progression into S phase, down-regulates the estrogen receptor, and up-regulates the progesterone receptor (7, 8). Furthermore, the exposure of MCF-7 cells to TNF-α for more than 3 h results in sustained expression of TNF-α mRNA and secretion of TNF-α protein (7). This indicates that TNF-α can regulate cytokine secretion in human breast tumor cells and raises the possibility that the secretion of other cytokines or growth factors could be modulated as well.

Human breast cancer cells secrete TGF-β, a growth-inhibitory protein. A variety of cell types, including epithelial cells, endothelial cells, fibroblasts, and lymphoid cells, have receptors for, and are responsive to, TGF-β. Thus, an agent such as a cytokine, which increases secretion of TGF-β from tumor cells, may have the potential for regulating tumor growth in an autocrine, paracrine, or even endocrine manner. It has been shown, for example, that the antiestrogens tamoxifen and LY117018 (9), progestins (10, 11), and the cytokine IL-1 (12) all induce secretion of TGF-β from estrogen receptor-positive breast cancer cells. IL-1 is of particular interest because it has many biological properties that overlap with those of TNF-α (13). Previously, we have shown that IL-1 stimulates secretion of TGF-β in a time- and dose-dependent manner in MCF-7 cells (12). The secreted TGF-β acts in an autocrine manner and mediates IL-1 inhibition of cell growth. Both TNF-α and IL-1 act through cell surface receptors, although TNF-α has greater growth-inhibitory and antiestrogenic effects on breast cancer cells (7, 8). This suggests that TGF-β may also be an important mediator of TNF-α action in breast cancer cells. In addition, there is evidence that TNF-α and TGF-β act together to regulate growth and metabolism in other malignant cells. In both HL60 and U937 leukemic cells, TNF-α and TGF-β act synergistically to inhibit cell proliferation and to induce monocytic differentiation, and TNF-α up-regulates TGF-β receptors in HL60 cells (14). Studies on the effect of TNF-α on TGF-β secretion by malignant cells are thus needed to clarify the interaction of these two cytokines.

In the present report, we show that TNF-α increases secretion of TGF-β in human breast cancer cells but not in immortalized normal mammary epithelial cells. Because TGF-β is secreted in both active and latent forms and as one of the three isomers TGF-β1, TGF-β2, or TGF-β3, we further characterized the effect of TNF-α on the TGF-β secretory pattern. Our findings are summarized below and indicate that the cytokine TNF-α is an important regulator of TGF-β secretion.
Stimulation of IGF-3 Secretion by INF-α

Materials and Methods

Materials

Leupeptin and aprotinin were purchased from Sigma Chemical Co. (St. Louis, MO). Purified human TNF-α (specific activity, 27 x 10^6 units/mg protein) was kindly provided by Cetus Corp. (Emeryville, CA). Purified human TGF-β1, anti-TGF-β neutralizing antibody, and affinity-purified rabbit polyclonal anti-TGF-β antibody for immunoblotting were obtained from R & D Systems (Minneapolis, MN). Polyclonal antisera specific for TGF-β1 or TGF-β2 and control sera were kindly given to us by Dr. Michael Sporn (Laboratory of Chemoprevention, National Cancer Institute, Bethesda, MD). ^125I-labeled TGF-β1 (specific activity, 2000–4500 Ci/mmol) and [3H]thymidine (specific activity, 86.1 Ci/mmol) were obtained from New England Nuclear (Boston, MA). The 40-mer oligonucleotide probes for TGF-β1, TGF-β2, TGF-β3, and β-actin that were used for Northern analysis were obtained from Oncogene Science (Cambridge, MA).

Cell Culture

MCF-7 cells were kindly provided by Dr. Marvin Rich (Michigan Cancer Foundation, Detroit, MI). T47D and ZR-75-1 breast cancer cells, 184B5 and MCF-10A normal mammary epithelial cells, A549 human lung carcinoma cells, and CCL-64 mink lung epithelial cells were obtained from the American Type Culture Collection (Rockville, MD). MCF-7 cells, T47D, ZR-75-1, and A549 cells were maintained in IMEM; CCL-64 cells were maintained in DMEM (Biofluids, Rockville, MD); and MCF-10A and 184B5 cells were maintained in mammary epithelial growth medium medium (Clonetics, San Diego, CA) containing 10 mg/ml phenol red and supplemented with 5% FCS, 2 mM glutamine, and 40 mg/liter gentamicin. Cells were maintained in a humidified atmosphere of 95% O2/5% CO2 at 37°C. Cell growth studies were conducted under these media conditions.

For metabolic studies and Northern analysis, unless otherwise specified in the figure legend, cells were plated in Costar flasks in IMEM without phenol red but supplemented with 2 mM glutamine, 40 mg/liter gentamicin, 15 IU/ml insulin, and 5% heat-inactivated calf serum stripped of endogenous steroids with dextran-coated charcoal (15). After 24 h, the monolayer was replaced with serum-free IMEM [2 µg/ml transferrin (Sigma), 1 µg/ml human fibronectin (Collaborative Research), 1 × trace element mix (Life Technologies, Inc., Grand Island, NY), 10 mM HEPES buffer (Life Technologies, Inc.), and 10 mM glutamine (Biofluids); Ref. 16], to which test substances were added. TNF-α was added from a stock concentrate maintained in HBSS.

TGF-β Determinations

Radioreceptor Assay. Medium was collected and centrifuged, and 0.2 trypsin inhibitor units/ml of aprotinin, 3 µg/ml leupeptin, and 1 mg/ml serum bovine albumin were added; then the samples were either assayed at that time or frozen at −70°C for subsequent assay. Samples were analyzed with or without prior acidification by the A549 lung carcinoma cell radioreceptor assay as follows (17). Medium (1.5 ml) was acidified to pH ≤2.0 with 6 N HCl, neutralized to pH 7.0 with 6 N NaOH/1.0 M HEPES (1:1 v/v), and concentrated 20–30-fold with a Centricon 10 concentrator (Amicon Corp., Danvers, MA) at 5900 RPM for 50 min. The concentrate was analyzed by radioreceptor assay according to the method of Danielpour et al. (17) as described previously (12). A parallel standard curve was performed using purified human TGF-β at concentrations of 0.01–10 ng/ml. Untreated serum-free medium served as a control, and treated samples were corrected for this value. Values were expressed as nanograms of TGF-β/10^6 cells of the feeder monolayer.

CCL-64 Mink Lung Growth Inhibition Assay. Serum-free medium was collected; protease inhibitors were added and either concentrated 20–30-fold directly or acidified, neutralized, and then concentrated, as described above. The [3H]thymidine growth inhibition assay was performed according to the method of Danielpour et al. (17). The concentrations of TGF-β in each sample were determined from a parallel standard curve and expressed as nanograms of TGF-β/10^6 MCF-7 cells of the feeder monolayer.

Cellular Extraction of TGF-β. The cell monolayers of T150 flasks were incubated with vehicle or TNF-α (1000 units/ml) for 24 h. Cells were harvested in PBS/0.04% EDTA, separated, and resuspended in 95% ethanol-concentrated HCl (375 ml:7.5 ml, v/v) supplemented with 1 mM phenylsulfonylurea and 3 µg/ml leupeptin. Cells were sonicated, adjusted to 6 ml/g tissue with distilled water, and extracted overnight at 4°C according to the method of Davoren (18) as modified by Roberts et al. (19). The sonicate was centrifuged, the supernatant was saved, and the pellet was resuspended in 95% ethanol/distilled water/concentrated HCl (375 ml:105 ml:7.5 ml v/v) and extracted for 2 h at 4°C. The extraction mixture was centrifuged, and the supernatants were combined and acidified to pH 5.2 with concentrated ammonium hydroxide, followed by the addition of 2 M ammonium acetate buffer (pH 5.3; 1 ml/85 ml extract). Two volumes of cold anhydrous ethanol and 4 volumes of cold anhydrous ether were added, and the mixture was allowed to stand for 48 h at −20°C. The mixture was centrifuged, the precipitate was dissolved in 1.0 M acetic acid and recentrifuged, and the supernatant was dialyzed against 0.17 M acetic acid in Spectropor 3 dialysis tubing (molecular weight cutoff, 3500; Thomas, Inc., Swedesboro, NJ) for 48 h at 4°C. The dialysate was then lyophilized, resuspended in binding buffer, and analyzed in the A549 radioreceptor assay as described above.

Neutralizing Antibody Studies. Isomeric TGF-β Studies. Serum-free IMEM from cells treated with vehicle or TNF-α (1000 units/ml for 72 h) was acidified, neutralized, and concentrated as described above. Aliquots were tested for the effect of polyclonal antisera specific for TGF-β1 or TGF-β2 (kindly provided by Dr. Michael Sporn) on the biological activity of these isoforms using the CCL-64 assay according to the method of Danielpour et al. (17). Dilutions of test samples were used to allow complete neutralization by a 1:250 dilution of specific antisera. The medium content of TGF-β was calculated from a parallel standard curve.

Cell Growth Studies. MCF-7 cells were plated in T25 flasks (10,000 cells/flask). After 24 h, medium was replaced with fresh complete medium containing vehicle, 1000 units/ml TNF-α, and/or 60 µg/ml TGF-β-neutralizing antibody (R & D Systems, Minneapolis, MN). Cultures were maintained in a humidified atmosphere of 95% O2/5% CO2 at 37°C.
Northern Analysis

Systems; a polyclonal antibody that neutralizes the activity of TGF-β1, TGF-β1, TGF-β2, TGF-β3, and TGF-β4 as determined by the manufacturer; this antibody has been shown previously to neutralize in vitro TGF-β secreted by these MCF-7 cells; Ref. 12). Normal rabbit IgG (R & D Systems) was used as control. Medium was replaced every 3 days, and cell counts were made on day 7. Studies were also conducted on cells incubated in serum-free or supplemented phenol-red-free IMEM, and cell counts were made after 3 and 7 days.

Immunoblotting

Serum-free medium from control or TNF-α-treated cells was collected and centrifuged, and aprotinin (0.2 trypsin inhibitor units/ml) was added to the supernatant. Aliquots (3.5 ml) were dialyzed in Spectropor 3 membranes against 0.2 M acetic acid for 48 h, as described by Gentry et al. (20). The dialysate was lyophilized and resuspended in Tris-glycine SDS sample buffer (Novex Corp, San Diego, CA) with or without 50 mM DTT. Samples were heated at 95°C for 3 min and electrophoresed in a SDS-polyacrylamide gel. Proteins were electrophotoreti
cally transferred to nitrocellulose filters and incubated in blocking solution (1 m glycine, 5% dry milk, 5% FCS, 1% grade II chicken egg ovalbumin, and PBS; pH 7.4) for 30 min at room temperature. Filters were then washed in a solution containing 1% FCS, 0.1% Tween 20, 0.1% dry milk, 0.1% grade III crystalline chicken egg ovalbumin, and PBS (pH 7.2) three times for 5 min each. Filters were then incubated overnight in wash solution containing affinity-purified rabbit polyclonal anti-TGF-β antibody (1.0 μg/ml; R & D Systems). Filters were washed with wash solution, followed by Tween-PBS [TPBS; PBS (pH 7.2) and 0.05% Tween 20] for 15 min, and then incubated with TPBS containing 1.0 μg/ml goat antirabbit IgG horseradish peroxidase conjugate (Bio-Rad, Hercules, CA) in the dark at room temperature for 60 min. The filter was washed with TPBS, exposed to enhanced chemiluminescence detection reagents (Amersham Corp, Arlington Heights, IL) for 60 s, and developed with Kodak X-OMAT film.

Gel Filtration Chromatography

Serum-free medium (80 ml) from control or TNF-α-treated cells was concentrated, dialyzed against 1 M acetic acid in Spectropor 3 dialysis tubing for 48 h, and lyophilized as described above for immunoblotting. The lyophilized medium was resuspended in 6 ml of 1 M acetic acid/PBS (1:3 v/v) and applied to a 57 x 2.6-cm Sephacryl S-100 HR gel filtration column (Pharmacia-LKB, Piscataway, NJ) preequilibrated with PBS (pH 7.4). Samples were eluted with PBS at room temperature, and 6-ml fractions were collected at a flow rate of 72 ml/h. Two-ml aliquots were concentrated 40-fold in Centricron 10 concentrators (Amicon Corp., Danvers, MA), and the TGF-β biological activity of each was determined in the CCL-64 mink lung assay as described above. The column was calibrated with chymotrypsin (M, 156,000), albumin (M, 67,000), and RNase A (M, 13,700) markers from Pharmacia-LKB.

Northern Analysis

Total cellular RNA was extracted by the RNAzol B method (Tel-Test, Friendswood, TX), and polyadenylated mRNA was selected with the Polytract mRNA Isolation System (Promega, Madison, WI). Five- to 10-μg samples were electrophoresed on a 1.2% agarose/formaldehyde gel as described elsewhere (21), transferred to nitrocellulose by positive pressure, and incubated overnight at 37°C in prehybridizing solution (21), followed by hybridizing solution (prehybrid + 10% dextran sulfate) containing 500,000 cpm/ml of 32P end-labeled TGF-β probe. Incubation was continued overnight at 37°C; the filter was washed with 1 x SSC (150 mM NaCl, 15 mM sodium citrate)/0.1% SDS followed by 0.25 x SSC/0.1% SDS at room temperature, and exposed to Kodak X-OMAT film at −70°C. Bound probe was then eluted with dH2O containing 1% glycerol at 80°C for 2 min (22), and filters were rehybridized with 32P end-labeled TGF-β1, TGF-β2, or β-actin probes. Blots were developed with Kodak X-OMAT film. Densitometry was performed on hybridized blots with a β-particle imager (Betascope 603 blot imager; Betagen, Waltham, MA). The transcript activity for the TGF-β and actin transcripts was measured in the control and TNF treatment groups for each blot, and the following densitometric ratio was determined:

\[ \frac{\text{TGF-β}_{\text{NSD}}/\text{Actin}_{\text{NSD}}}{\text{TGF-β}_{\text{CONTROL}}/\text{Actin}_{\text{CONTROL}}} \]

Statistical Determinations

Statistical comparisons were analyzed by nonpaired Student’s t test (23) or by one-way ANOVA (24).

Results

Effect of TNF-α on Breast Cell Line Growth and TGF-β Secretion. We first examined the effect of TNF-α (1000 units/ml) on cell growth and TGF-β secretion of three breast cancer cell lines (MCF-7, ZR-75-1, and T47D) and two immortalized mammary epithelial cell lines (184B5 and MCF-10A). MCF-7 and ZR-75-1 cell lines were growth inhibited by TNF-α (75.8 and 75.7% inhibition, respectively; Fig. 1A); T47D, 184B5, and MCF-10A cell lines were resistant to TNF-α (16.5, 10.3, and 5.2% inhibition, respectively). The degree of cytotoxicity for MCF-7 cells has been shown previously to be 0.0, 0.0, and 40.0%, at 24, 48, and 72 h, respectively, of TNF treatment (8).

Four of the five cell lines that were examined for growth response (MCF-7, ZR-75-1, T47D, and 184B5) secreted detectable levels of TGF-β (Fig. 1B). TNF-α increased secretion of TGF-β in MCF-7 and ZR-75-1 cells by 5.1- and 2.8-fold, respectively, over basal levels. TNF-α had no effect on TGF-β secretion by the resistant cell lines T47D, 184B5, and MCF-10A. Cell growth and TGF-β secretion studies, taken together, show that TNF-α enhances TGF-β secretion in those cell lines that are growth inhibited by TNF-α, but TNF-α has no effect on TGF-β secretion in unresponsive cell lines.

MCF-7 cells were selected for further study because of the pronounced induction of TGF-β by TNF-α and because of our previous studies in which we characterized the growth and metabolic responses of these cells to TNF-α (8). Serum-free medium incubated with control MCF-7 cells for 72 h contained 3.3 ± 0.6 ng total TGF-β/106 cells, as determined by the radioreceptor assay (Fig. 2). TGF-β medium content in the
Fig. 1 Effect of TNF-α on cell growth and TGF-β secretion by breast cell lines. A monolayer of cells of the indicated cell line was incubated with vehicle (C) or TNF-α (T; 1000 units/ml) in phenol red complete medium supplemented with 5% FCS, and the cell count was made on day 3. Each value is expressed as the mean percentage of inhibition by TNF-α versus control for two to four experiments in duplicate (A); bars, SE. *versus control and T47D, 184B5, and MCF-10A; P < 0.01. The medium was analyzed for TGF-β content after acid activation by the radioreceptor assay, as described in “Materials and Methods” (B). Each value in B is expressed as nanograms of TGF-3/10⁶ cells of the feeder monolayer and represents the mean for two to four experiments in duplicate; bars, SE. *versus respective control; P < 0.01.

Absence of acid activation was 0.1 ± 0.04 ng/10⁶ cells, indicating that approximately 96% of TGF-β secreted by control cells is in the latent form. The acid-activated TGF-β was biologically active, as determined by the CCL-64 mink lung assay (Fig. 3). The nonacid activated medium contained a lower but detectable quantity of biologically active TGF-β.

Effect of TNF-α on TGF-β Secretion in MCF-7 Cells. Exposure of MCF-7 cells to TNF-α in serum-free medium for 72 h enhanced the secretion of total TGF-β from 3.3 to 20.0 ng/10⁶ cells (6.1-fold; Fig. 2). TNF-α stimulation of TGF-β secretion was dose dependent, with a significant increase at 100 units/ml. TNF-α stimulation of TGF-β was time dependent, with a significant increase noted by 24 h (Fig. 5). Stimulation by TNF-α progressed linearly and was greater at 72 and 96 h than at 48 h. The addition of TNF-α to the assay medium of untreated cells did not alter the measured TGF-β content, indicating that TNF-α is not simply interfering with the binding of 125I-labeled TGF-β to the cells in this assay.

The secreted levels of TGF-β in these studies were expressed as nanograms per 10⁶ cells. When expressed as nanograms per 4 ml medium/flask, the TGF-β level in the medium for TNF-α-treated cells was 5.4 ± 0.6 ng/flask compared to control medium, which was 1.8 ± 0.2 ng/flask (P < 0.01). Therefore, TNF-α is acting to increase the net medium content of this growth factor. We have shown previously that cells exposed to TNF-α (1000 units/ml) for 48 h were 100% viable (8). To test the possibility that TNF-α is acting to release intracellular pools of TGF-β, we studied the effect of TNF-α on...
TNF-α to the medium of control CCL-64 cells had no effect on the intracellular content of TGF-β. We found that the TGF-β content of the TNF-α treated cells (1.2 ± 0.3 ng/mg DNA) was not significantly different from that of the control cells (1.7 ± 0.8 ng/mg DNA). TNF-α is, therefore, not acting to simply reduce or redistribute the intracellular pools of TGF-β.

TNF-α increased the secretion of both latent and active forms of TGF-β (Figs. 2 and 3). TNF-α induced a shift in the ratio of latent:active forms in the medium from 25:1 for controls to 12:1 for TNF-α-treated cells. The addition of exogenous TNF-α to the medium of control CCL-64 cells had no effect on [3H]thymidine uptake by CCL-64 cells. Any residual TNF-α in the test samples was thus not interfering with the measurement of active TGF-β in these assays. To confirm that the increase in active TGF-β in the media by TNF-α was also not a direct effect of TNF-α on the medium proteins, the effect of TNF-α on extracellular degradation of TGF-β was examined. To the medium of a monolayer of cells was added 100 pm of 125I-labeled TGF-β and vehicle or TNF-α (1000 units/ml). The acid-precipitable radioactivity of the medium was measured at 1-h intervals over 6 h, according to the method of Frolik et al. (25). No differences were noted at any of these time points between the two groups in precipitable 125I-labeled TGF-β. This indicates that TNF-α is not acting to stabilize TGF-β in the media of these cells.

**Effect of TNF-α on Secretion of TGF-β Isomers.** The specificity of the isomeric forms of TGF-β in the media of control and TNF-α-treated cells was determined with polyclonal specific antisera. Specificity of the antisera for TGF-β1, TGF-β2, and/or TGF-β3 respectively, has been described elsewhere (17). Within the control group, TGF-β activity was completely neutralized by anti-TGF-β1 antiserum but was only partially neutralized by anti-TGF-β2 antiserum (Table 1). The sum of the inhibition by the two antibodies (183.5%) greatly exceeded 100% and indicated that untreated MCF-7 cells secrete predominantly the heterodimeric form TGF-β1-2, with a smaller amount of homodimeric TGF-β1. When used in combination, anti-TGF-β1 + anti-TGF-β2 completely neutralized the TGF-β activity of the control media, indicating that the other isomeric form (TGF-β3), if present, does not contribute to the inhibitory activity of the medium.

The medium from the TNF-α-treated cells was partially neutralized by anti-TGF-β1 (41.6%) and partially neutralized by anti-TGF-β3 (91.2%). If present, does not contribute to the inhibitory activity of the medium.

**Immunoblot of TGF-β Secreted by MCF-7 Cells.** We examined acid-activated media from control cells by immuno-

![Figure 4](image_url) **Fig. 4** Effect of TNF-α concentration on TGF-β secretion. A monolayer of cells was exposed to vehicle (C) or TNF-α (T; 1000 units/ml) for the indicated time periods. The medium was analyzed for TGF-β content by the radioreceptor assay after acid activation, as described in “Materials and Methods.” Values are the means for three experiments in duplicate; bars, SE. *versus control; P < 0.05.

![Figure 5](image_url) **Fig. 5** Time course of TNF-α stimulation of TGF-β secretion. A monolayer of cells was exposed to vehicle (C) or TNF-α (T; 1000 units/ml) for the indicated time periods. The medium was analyzed for TGF-β content by the radioreceptor assay after acid activation, as described in “Materials and Methods.” Values are the means for three experiments in duplicate; bars, SE. *versus control; P < 0.05.

### Table 1: Effect of TNF-α on isomeric TGF-β secretion

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Inhibition of TGF-β activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C + anti-TGF-β₁</td>
<td>95.4% ± 0.9</td>
</tr>
<tr>
<td>T + anti-TGF-β₁</td>
<td>41.6% ± 13.4*</td>
</tr>
<tr>
<td>C + anti-TGF-β₂</td>
<td>88.1% ± 6.2</td>
</tr>
<tr>
<td>T + anti-TGF-β₂</td>
<td>67.1% ± 9.3</td>
</tr>
<tr>
<td>C + anti-TGF-β₁/TGF-β₂</td>
<td>99.2% ± 0.7</td>
</tr>
<tr>
<td>T + anti-TGF-β₁/TGF-β₂</td>
<td>91.2% ± 2.7</td>
</tr>
</tbody>
</table>

*P < 0.01 vs. C + anti-TGF-β₁.
Gel Filtration Analysis of TGF-β Secreted by MCF-7 Cells. Gel filtration analysis of acid-activated media indicated that MCF-7 cells secrete multiple biologically active forms of TGF-β. The profiles for control and TNF-α-treated media are illustrated in Fig. 7. The molecular mass species identified by gel filtration included 90, 69, 41, 24, and 12 kDa. This, with the exception of the 69-kDa form, is consistent with the findings by gel electrophoresis. Knabbe et al. (9) also noted a 69-kDa form by gel filtration using acetic acid and nonreducing conditions but not by immunoprecipitation. The 69-kDa form could represent aggregates of smaller forms. These findings indicate that the 44-kDa precursor protein and the 88-kDa protein, both of which contain the mature TGF-β protein (demonstrated by immunoblotting), are biologically active forms of TGF-β. TNF-α treatment did not cause any change in the distribution of biologically active TGF-β proteins secreted by MCF-7 cells (Fig. 7).

Effect of TNF-α on TGF-β mRNA Levels. Control MCF-7 cells contained mRNA transcripts for TGF-β₁, TGF-β₂, and TGF-β₃ (Fig. 8). Exposure to TNF-α did not significantly alter steady-state levels of mRNA transcripts for TGF-β₁ or TGF-β₂ (densitometric ratio, 1.1 ± 0.16 and 1.2 ± 0.35, respectively, for three or four Northern blots) and caused a modest reduction in TGF-β₃ (densitometric ratio, 0.43 ± 0.12). No new transcripts were induced by TNF-α.

Effect of TGF-β Neutralizing Antibody on Cell Growth. The effect of neutralizing antibody against TGF-β on the cell growth of control and TNF-α-treated cells was studied. Exposure of control cells to TGF-β neutralizing antibody (60 µg/ml) was associated with partial augmentation of growth (Fig. 9). This result is consistent with previous studies indicating that
TGF-β secreted by these control MCF-7 cells acts in an autocrine manner to inhibit cell growth (12). Neutralizing antibody to TGF-β did not reverse TNF-α (1000 units/ml) inhibition in either serum-free media or in supplemented IMEM after 3 or 7 days. This indicates that TNF, although enhancing TGF-β secretion, blocks TGF-β autocrine inhibition of growth of these cells.

Discussion

In this study, we show that TNF-α selectively increased the secretion of TGF-β from human breast cancer cells in vitro. The effect of TNF-α on TGF-β secretion was rapid, involved both latent and active forms of TGF-β, and correlated with the ability of TNF-α to inhibit cell growth. TNF-α had no effect on TGF-β secreted by two immortalized mammary epithelial cell lines or on one breast cancer cell line that was not growth inhibited by TNF-α. In addition, we found that TNF-α did not act extracellularly to stabilize TGF-β or to interfere with the turnover of the latent complex and did not release stored intracellular pools of TGF-β. TNF-α is thus actively regulating the metabolism of TGF-β. These findings further support an important interaction between TNF-α and TGF-β in malignant cells. Previous studies have shown that TNF-α can increase TGF-β receptors and can act synergistically with TGF-β to inhibit cell proliferation and to induce monocyte differentiation in HL60 and U937 leukemia cells (14). TNF-α can thus regulate secretion, binding, and cellular response to TGF-β in malignant cells.

In human breast cancer cells, TNF-α increased the secretion of both latent and active forms of TGF-β. This could have important implications for the control of cell growth and metabolism. Secretion of the latent forms of TGF-β was increased 5.8-fold by TNF-α. The latent form constituted 91.4% of total (latent and active) TGF-β in the medium. The latent form of TGF-β has a prolonged half-life and greater volume of distribution than the active form (26). In addition, the latent complex does not bind to TGF-β receptors and may be selectively targeted to extracellular sites. Heldin et al. (27) have presented evidence that the latent form is selectively targeted to the extracellular matrix by the latent TGF-β-binding protein, where it is activated by plasmin. TNF-α-enhanced secretion of the TGF-β latent form may thus allow TNF-α to act in an endocrine fashion by regulating cellular activities over a longer period of time and at more distant sites.

TNF-α is an important addition to the group of compounds that increase TGF-β secretion and inhibit the growth of breast cancer cells. This group includes tamoxifen (9) and IL-1 (12). These compounds, like TNF-α, act predominantly on estrogen receptor-positive cells and have in common the capacity to rapidly increase TGF-β secretion. TNF-α differs, however, from these compounds in several respects. TNF-α is a more potent inhibitor of growth than is tamoxifen or IL-1. IL-1 and tamoxifen inhibition of growth is mediated by TGF-β, whereas TNF-α inhibition is not. Tamoxifen acts through nuclear estrogen receptors that are transcription factors, whereas TNF-α and IL-1 act through cell surface receptors. Among breast cancer cells, tamoxifen increases secretion of the isomer TGF-β2 from MCF-7 cells (10), whereas TNF-α increases secretion of the isomer TGF-β1. These differences indicate the complexity of events regulating TGF-β secretion and may reflect the different signal transduction pathways used by TNF-α compared with tamoxifen and IL-1.

TNF-α had two important effects on the secretion of isomeric TGF-β: (a) TNF-α increased secretion of TGF-β2; and (b) TNF-α altered the dimeric pattern of TGF-β secretion from one predominantly of the heterodimer TGF-β1,2 to one predominantly, if not exclusively, of the homodimers TGF-β1 and TGF-β2. Other substances [tamoxifen (10), retinoid acid (28), calcium (29), and epidermal growth factor (30)] have also been shown to regulate the secretion of specific isomers of TGF-β. The present study, however, is the first report of a cytokine regulating TGF-β isomeric secretion in breast cancer cells. MCF-7 cells are 5-10-fold less sensitive to TGF-β2 than to TGF-β1 (31). The shift in secretory pattern from TGF-β1,2 to...
TGF-β could, therefore, potentially reduce the autocrine inhibitory effect of TGF-β on these cells. In fact, we found that neutralizing antibody to TGF-β blocked the inhibitory effect of TGF-β secreted by control cells but not by cells treated with TNF-α. We have not examined the effect of TNF-α on the TGF-β receptor of MCF-7 cells, but our findings indicate that TNF-α modifies the autocrine response of the cell to TGF-β.

The total TGF-β secreted by MCF-7 cells is composed of multiple molecular mass species of TGF-β. Under nonreducing conditions, four molecular mass species were identified: 238, 210, 40–55, and 25 kDa. Under reducing conditions, 88-, 44-, and 12-kDa forms were noted. The 25-kDa form identified under nonreducing conditions represented the active dimeric form consisting of two 12-kDa subunits bound covalently through disulfide linkage. Neutralization experiments (Table 1) indicated that the dimeric form in the control cells represented predominantly the heterodimer TGF-β12. The active subunits of TGF-β1 and TGF-β2 are both 112 amino acids; therefore, identical electrophoretic migration of the two subunits would be expected under reducing conditions. Gel filtration identified multiple biologically active species secreted by MCF-7 cells. The 12- and 25-kDa peaks represent the monomeric and dimeric mature forms, respectively. The 41-kDa peak is in good agreement with the 44-kDa species identified by gel electrophoresis and is consistent in size with the (nonproteolytically cleaved) precursor form.

TGF-β secretion of multiple forms with different biological activities may also represent an important means by which exogenous substances control the activity, timing, and distribution of TGF-β activity. The latent complex is not biologically active and has a longer half-life and volume of distribution than the mature forms. The precursor peptide appears to have an "intermediate" level of activity between latent and mature forms. The heterodimeric mature form TGF-β12, with isomers TGF-β1 and TGF-β2, which have different biological activities, further increases the broad spectrum of TGF-β activities. Because a wide variety of cell types are responsive to TGF-β, this broad spectrum of forms may provide a better control over the actions of cells by TGF-β. At the same time, the spectrum of TGF-β forms provides more potential sites for control of TGF-β secretion by exogenous substances such as TNF-α.

Several findings in the present study clarify the mechanism of action of TNF: (a) TNF did not act to release intracellular pools of TNF and did not act to stabilize extracellular TGF-β; (b) TNF did not alter the molecular mass profiles of TGF-β secreted by these cells, and no new larger or smaller molecular mass species were observed after TNF treatment, as indicated by immunoblotting or gel filtration studies; and (c) TNF did not alter steady-state levels of mRNA for TGF-β1 or TGF-β2, corresponding to the major TGF-β protein forms secreted by these cells. No new mRNA transcripts were noted in TNF-α-treated cells. These findings suggest that TNF-α regulation of TGF-β1 and TGF-β2 secretion probably does not take place at the transcriptional level; however, posttranscriptional control and/or altered translational efficiency remain possible mechanisms of action. Current studies in other laboratories have indicated that retinoic acid, for example, may be regulating TGF-β secretion through its actions on the 5' untranslated region of the transcript (33). It would be of interest to examine this as a possible site of action for TNF-α.

In summary, these finding show that TNF-α qualitatively and quantitatively increases secretion of TGF-β in human breast cancer cells. This effect correlates directly with growth inhibition by TNF-α and indicates perhaps additional pathways regulating TGF-β metabolism. Enhanced secretion of TGF-β by TNF-α allows TNF-α to influence other cell types in a breast malignancy and may provide important insight into the mechanism of action of this cytokine.

References


Tumor necrosis factor alpha enhances secretion of transforming growth factor beta2 in MCF-7 breast cancer cells.

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