Growth Stimulation of Human Breast Cancer Cells with Anti-Transforming Growth Factor β Antibodies: Evidence for Negative Autocrine Regulation by Transforming Growth Factor β

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Abstract
Exogenous TGFβ inhibits the proliferation of human breast cancer cells in vitro. These cells synthesize and secrete TGFβ into their medium predominantly in a latent form. With neutralizing antibodies against native, biologically active TGFβ (278ab and 282ab), we have examined whether HS578T and MDA-231 breast cancer cells utilize their endogenous TGFβ for growth regulation. Neutralizing antibody activity was detectable in conditioned medium from confluent monolayers of both cell lines in the absence of acid or protease treatment as measured by radioreceptor assay. When added to subconfluent monolayers of the respective cell line, this untreated conditioned medium inhibited DNA synthesis and cell proliferation. This inhibition was blocked by anti-TGFβ antibodies, whereas nonimmune rabbit IgG had no effect. Similar to exogenous TGFβ1, this conditioned medium induced a dose-dependent increase in steady-state TGFβ1 mRNA levels when added to subconfluent HS578T cells; this increase was blocked by the 278ab. Consistent with the above, preincubation of either cell line with anti-TGFβ antibodies increased subsequent specific binding of [3H]TGFβ to cell surface receptors without changing binding affinity. Addition of 278ab to quiescent HS578T or MDA-231 cells induced a dose-dependent increase in [3H]thymidine incorporation. Both antibodies stimulated cell proliferation in serum-free medium and anchorage-independent growth of both cell lines. Finally, incubation of HS578T cells with 278ab under serum-free conditions decreased the basal level of TGFβ1 message expression. These data indicate that cultured human breast cancer cells utilize endogenously produced TGFβ as an autocrine negative growth regulator.

Introduction
TGFβ is a member of a multifunctional family of polypeptides first identified for the ability to induce anchorage-independent growth of nontransformed fibroblasts (1). It is a potent inhibitor of normal epithelial cells and of some neoplastically transformed epithelial cells (2–4). It has recently been established that there are at least three TGFβ mammalian proteins, each coded by different genes (5). Although minor differences in potency exist among these species, in general they exhibit similar effects in a variety of biological assays (5).

Previous studies have reported that human breast cancer cells are inhibited by picomolar concentrations of exogenous TGFβ1 and TGFβ2, express cell surface receptors for this ligand, express TGFβ mRNA, and secrete TGFβ activity (6–12). Knabbe et al. (9) reported that estrogen-dependent MCF-7 breast cancer cells, when exposed to inhibitory concentrations of antiestrogens, secreted 8–27-fold higher levels of TGFβ1 protein without associated changes in TGFβ1 mRNA expression. A more recent study reported that TGFβ2 and β3 mRNA levels, but not the TGFβ1 mRNA level, are decreased by estradiol in some hormone-responsive breast cancer cells (13). Others have shown high constitutive levels of TGFβ activity in media from estrogen-independent highly tumorigenic breast cancer cells (6, 14). Cultured normal mammary epithelial cells also express high levels of TGFβ1 mRNA and are sensitive to TGFβ-induced inhibition (15). In vivo, local administration of TGFβ caused reversible inhibition of developing mouse mammary glands (16). Taken together, these data suggest a functional role for TGFβ in breast epithelial cell biology.

Studies with cultured fibroblasts have shown that secreted TGFβ is in a latent form detectable only after treatment with acid or proteases like plasin (17–19). Similar to these studies, the majority of the TGFβ activity secreted by breast cancer cells is in an inactive form. Whether the latent TGFβ can be activated, either spontaneously or by the secreting cells themselves, with subsequent binding to cell surface receptors and autocrine growth regulation is not known. The latency of TGFβ in cell-conditioned medium, however, does not exclude the possibility that the cells utilize some of the endogenous TGFβ as a growth-regulatory mechanism. This possibility was examined in the human breast cancer cell lines HS578T and MDA-231 utilizing neutralizing antibodies against mature, biologically active TGFβ. The results of these experiments suggest the presence of a functional TGFβ activity in breast cancer cell-conditioned medium that, in the absence of acid or protease treatment, can induce growth inhibition and autode induction of TGFβ1 mRNA expression. Since breast cancer cells are exquisitely sensitive to exogenous TGFβ, the possibility exists that activation of at least a portion of the large

367-374, August 1990 Cell Growth & Differentiation 367

Received 4/10/90.

1 This work was supported by Department of Veterans Affairs Career Development Awards (C. L. A. (R. J. C.), USPHS Grants CA 46413 (R. J. C.) and CA42572 (H. L. M.), and the Culepper Foundation (R. J. C.).
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3 The abbreviations used are: TGFβ, transforming growth factor β; cDNA, complementary DNA; IMEM, improved minimal essential medium.
amounts of latent TGFβ secreted by the cells may have important autoinhibitory effects against breast tumors in vivo.

**Results**

**Human Breast Cancer Cells Secrete Bioactive TGFβ.** We first examined the secretion of TGFβ activity into conditioned medium by hormone-independent MDA-231 and HS578T breast cancer cells. TGFβ activity was present in untreated conditioned medium as measured by a TGFβ radioreceptor assay (Table 1). Upon acidification, this activity increased 100–1000-fold. When added to subconfluent monolayers of the same cell line, the untreated as well as the transiently acidified conditioned medium induced dose-dependent inhibition of DNA synthesis (Fig. 1). In both cases, this inhibition was blocked with antibodies against mature TGFβ, whereas nonimmune rabbit IgG had no effect (Fig. 1). Similar results were observed when growth in monolayer was examined (data not shown). When an inhibitory concentration of acid-treated conditioned medium was kept constant, the 282ab reversed the medium-induced inhibitory effect of subconfluent HS578T cells in a dose-dependent manner (Table 2).

**Secreted TGFβ Activity Autoinduces TGFβ1 Expression in HS578T Cells.** We next examined whether TGFβ1 is autoregulated in HS578T cells. In AKR-2B mouse fibroblasts and in mouse skin keratinocytes (BALB/MK), TGFβ1 mRNA is maximally autoinduced at 6–12 and at 12–24 h, respectively (20). Therefore, a TGFβ1 dose response on basal message levels at a single time was examined first. Exposure for 12 h to inhibitory doses of recombinant TGFβ1 increased steady-state levels of TGFβ1 mRNA. This autoinduction was half-maximal at approximately 0.25 ng/ml (10 pM) with maximal stimulation corresponding to a 4-fold increase in message levels (Fig. 2A). Using an optimal dose of 2.5 ng/ml, a time course of the autoinduction was performed. Accumulation of TGFβ1 message was detected within 1 h after TGFβ treatment; maximal induction (4–5-fold) was seen after 12–24 h and persisted 48 h later (Fig. 2B).

Since low levels of active TGFβ were detected in cell-conditioned medium without acid or protease treatment, we examined whether this medium would also autoinduce TGFβ1 message levels. Addition of untreated conditioned medium from confluent HS578T cells to subconfluent monolayers of HS578T cells induced a dose-dependent increase of TGFβ1 mRNA (Fig. 3). This induction of TGFβ1 expression was blocked by preincubation of the untreated conditioned medium with the 278 anti-TGFβ antibody but not with nonimmune rabbit IgG (Ta-

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**Table 1** TGFβ activity in breast cancer cell-conditioned medium

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TGFβ activity/10⁶ cells (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untrated</td>
</tr>
<tr>
<td>HS578T</td>
<td>1.7</td>
</tr>
<tr>
<td>MDA-231</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*Numbers in parentheses, concentration (ng/ml).

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**Table 2** Reversal of the conditioned medium-induced inhibition of DNA synthesis in HS578T cells with 282ab

<table>
<thead>
<tr>
<th>282ab (µg/ml)</th>
<th>Conditioned medium (fold)</th>
<th>cpm/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>7866 ± 1934</td>
</tr>
<tr>
<td>100</td>
<td>0.5</td>
<td>5479 ± 1462</td>
</tr>
<tr>
<td>25</td>
<td>0.5</td>
<td>4431 ± 1552</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>2477 ± 194</td>
</tr>
<tr>
<td>2.5</td>
<td>0.5</td>
<td>910 ± 183</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>667 ± 198</td>
</tr>
<tr>
<td>0</td>
<td>0.5</td>
<td>589 ± 91</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Effect of conditioned medium from confluent HS578T or MDA-231 cell monolayers on DNA synthesis in subconfluent HS578T or MDA-231 cells. Medium conditioned for 48 h by HS578T or MDA-231 cells was added in different dilutions to subconfluent HS578T or MDA-231 cells, respectively, in 24-well plates. Conditioned medium was left untreated (■, □) or acidified and reneutralized as described in "Materials and Methods" (●, ○). Prior to addition to the monolayers, the different dilutions of conditioned medium were preincubated overnight at 4°C with 30 µg/ml of nonimmune rabbit IgG (●, ○) or 282ab (□, △). Tritiated thymidine incorporation was measured 18 h after the addition of conditioned medium. Each data point, mean of triplicate determinations. All SE were less than 10%.
for 24 h with the 278 anti-TGFβ antibody increased specific binding of labeled TGFβ predominantly to the type I receptor when compared to no pretreatment or to nonimmune rabbit IgG (Fig. 4). When quantitated by laser densitometry, the difference between uncompeted and competed labeled cell surface proteins was restricted to the type I receptor in both cell lines.

Scatchard analyses of competition binding experiments with both cell lines showed an antibody-induced increase in TGFβ receptors without a change in binding affinity. A representative Scatchard plot of MDA-231 and HS578T cells is shown in Fig. 5. The calculated receptor

KS was 45 pm for the MDA-231 and 27 pm for the HS578T line for both the antibody-treated and control IgG-treated cells. MDA-231 cells preincubated with the 278ab exhibited 3000 binding sites/cell, whereas those treated with the control IgG had only 1000 sites/cell. In the HS578T cells, there were 2000 and 1000 TGFβ binding sites, respectively (Fig. 5).

Anti-TGFβ Antibodies Stimulate Growth of Human Breast Cancer Cells. The ability of anti-TGFβ antibodies to modulate basal growth of both cell lines was tested. If the secreted TGFβ activity is functioning as a negative autocrine growth regulator in these cells, then antibody-induced blockade of the endogenous TGFβ may indirectly stimulate growth. In these experiments, HS578T cells became quiescent after 4–5 days in serum-free medium. Addition of 278ab to quiescent cells in the absence of serum induced a transient dose-dependent increase in DNA synthesis 18–24 h after antibody treatment (Fig. 6). Earlier time points did not show such an increase, and by 48 h, the antibody-treated cells became quiescent again. Similar results were observed with the MDA-231 cells (data not shown).

Finally, monolayer growth (Table 4) and colony formation in soft agarose of both cell lines (Fig. 7) were stimulated by anti-TGFβ antibodies in a dose-dependent fashion, whereas nonimmune rabbit IgG had no effect. These experiments were done at least twice with similar results. The anchorage-independent growth assays were the only ones done in the presence of serum. Thus, antibody-induced blockade of endogenous TGFβ enhanced mitogenicity and growth of human breast cancer cells.

Discussion

It has been proposed that TGFβ is an autocrine negative growth regulator in epithelial cells (1). Confirmation of this hypothesis, though, has not been documented. For our studies, blocking antibodies against mature TGFβ were used. These antibodies have been reported to inhibit spontaneous colony formation in soft agar of transformed AKR-MCA fibroblasts (21), suggesting that TGFβ secreted by the cells plays a role in anchorage-independent growth. Although this inhibition was reversed by exogenous TGFβ, this study could not determine whether the antibodies were blocking TGFβ released by the cells and/or contained in serum. In order to avoid the effect of TGFβ present in serum, our experiments were performed under serum-free conditions. These studies utilized the estrogen-independent human breast cancer cell lines HS578T and MDA-231. These cells are growth inhibited by exogenous TGFβ with a 50% inhibitory concentration of <10 pm (6, 12).

TGFβ activity was detectable in medium from con-

Fig. 2. Autoinduction of TGFβ1 mRNA expression in HS578T cells. A, rapidly growing HS578T cells in serum-free medium were incubated with different concentrations of TGFβ1. After 12 h, total cellular RNA was isolated and processed. B, HS578T cells were treated with 2.5 ng/ml (0.1 ng) TGFβ1, and at the indicated times, total cellular RNA was isolated and processed. In both cases, Northern blot analysis was performed with 2 μg of polyadenylated RNA using 32P-labeled TGFβ1 cDNA and 1B15 cRNA probes as described in “Materials and Methods.” Ctrl, control.
fluent cells in the absence of acid or protease treatment at concentrations consistent with inhibitory doses of exogenous peptide (Table 1). This conditioned medium was inhibitory to subconfluent monolayers of the respective cell line in a reversible fashion. Furthermore, in HS578T cells, it autoinduced steady-state TGFβ1 message levels. Since TGFβ activity as measured by radioreceptor assay did not change during the first 24 h (data not shown), and since only fresh medium was used in our experiments, it is unlikely that spontaneous activation was a major factor for our results. It is possible, however, that under certain conditions the secreting cells may activate latent TGFβ themselves. This possibility is supported by a recent report in which UV-induced fibrosar-
for TGFβ (6, 9, 12), we examined whether antibody blockade of the secreted TGFβ activity detected in conditioned medium would up-regulate receptor binding. In these cells, TGFβ1 and β2 are mutually competitive for receptor binding with similar affinity (12). Preincubation of both cell lines with anti-TGFβ antibodies increased specific binding of ¹²⁵I-TGFβ to cell surface receptors, especially the type I receptor. In other systems, binding to this receptor has been necessary to elicit TGFβ cellular responses (25). Furthermore, Mv1Lu mutants that have selectively lost expression of the type I receptor lose the ability to respond to TGFβ (26). The up-regulation of type I receptor binding with anti-TGFβ antibodies further argues that on individual cells mature secreted TGFβ interacts with surface proteins present in the same cells and that this endogenous ligand-receptor interaction results in growth inhibition. This was further supported by Scatchard analysis of competition binding experiments performed in high density cultures. Binding of TGFβ to MDA-231 and HS578T cells treated with the control IgG was markedly decreased (Fig. 5) when compared to previous studies with these cell lines (6, 12). This finding agrees with the reported high density-induced down-regulation of TGFβ binding on other cell lines (27). This, in turn, may reflect higher levels of secreted TGFβ activity in the confluent cultures. This down-regulation was prevented by preincubation with anti-TGFβ antibodies.

Supporting a functional role for this secreted ligand-receptor interaction, DNA synthesis in serum-starved quiescent cells and cell proliferation in serum-free medium were both consistently stimulated by the neutralizing TGFβ antibodies. The rather modest growth stimulation [20–135% above control (Fig. 7, Table 4)] may not be surprising since TGFβ is probably only one of several endogenous inhibitors of mammary epithelial cells (28–30).

The observation that some normal human bronchial cells are inhibited and induced to differentiate by TGFβ contained in serum (31) has suggested that benign epithelial cells are capable of activating the inactive form of TGFβ. On the other hand, many cancer cells grow optimally in serum but are inhibited by mature, active TGFβ (2, 3). These observations have led to the hypothesis that some malignant epithelial cells have lost the ability to activate endogenous TGFβ, resulting in loss of a normal growth-regulatory mechanism and a subsequent proliferative advantage that contributes to the neoplastic phenotype (32, 33). The large amount of activatable latent TGFβ secreted by the breast cancer cells in this study is consistent with the previous hypothesis. Correspondingly, the highly tumorigenic MDA-231 cells secreted less active TGFβ than the slower growing HS578T cells (Table 1). However, some constitutive activation would have had to take place in order to explain our results. Whether normal breast epithelial cells can activate TGFβ or whether the ratio of the active/latent secreted TGFβ is different from that in their transformed counterparts has not been investigated.

Taken together, these data suggest that human breast cancer cells in culture can utilize endogenously produced TGFβ as a negative growth regulator. Whether further growth control can be achieved by activating the large quantities of latent TGFβ secreted by the cancer cells remains to be studied. Twardzik et al. (34) recently reported inhibition of A549 human lung carcinoma cells in vivo by peritumoral subcutaneous inoculations of...
TGFβ1 or TGFβ2. Although this report is encouraging, future use of TGFβ as an antitumor agent will be hampered by the fact that, after systemic administration to animals, this molecule is rapidly cleared by the liver with a t½ of 2.2 min (35). One could speculate that an alternative would be to activate the large pool of latent TGFβ secreted by the cancer cells in order to induce autocrine growth inhibition. The potential usefulness (or toxicity) of this strategy in the malignant breast requires further investigation.

Materials and Methods

Cell Culture, Growth Factors, and Antibodies. The HS578T and MDA-231 human breast cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD) and passaged in improved minimal essential medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (Hazelton, Lenexa, KS). The AKR-2B cells were maintained in McCoy's 5a medium supplemented with 5% fetal calf serum. Recombinant human TGFβ1 was provided by Dr. Saied Seyedin (Celltrix Laboratories, Collagen Corp., Palo Alto, CA). In order to block native, biologically active TGFβ, the rabbit polyclonal antibodies 278 and 282 were utilized. These antisera were raised against platelet-derived porcine TGFβ (21); both antisera immunoprecipitate the M, 25,000 mature TGFβ, inhibit binding of 125I-TGFβ to cell surface receptors, and neutralize the inhibitory effect of TGFβ1 and TGFβ2 on AKR-2B cells (21). Nonimmune rabbit serum or normal rabbit IgG (Sigma, St. Louis, MO) was used as a control for the blocking antibodies.

Collection of Conditioned Medium. Cells were plated in 75-cm² tissue culture flasks in regular growth medium. When they were 50–75% confluent, the monolayers were washed twice with phosphate-buffered saline and changed to serum-free IMEM containing transferrin (2 mg/liter; Sigma). The conditioned medium was collected 48 h later, and leupeptin (1 µg/ml; Sigma) was added. The medium was centrifuged at 3000 rpm for 10 min in order to remove cellular debris and stored at 4°C in order to prevent spontaneous activation. When indicated, the medium was acidified to pH 1.5 for 1 h with HCl and renaturated with NaOH before use. The conditioned medium was utilized within 6 h of collection to avoid prolonged storage.

TGFβ Binding Studies. TGFβ radioreceptor assays were performed as previously described (19) utilizing AKR-2B (clone 8A1) cells as indicator cells. Porcine TGFβ2 (R & D Systems, Inc., Minneapolis, MN) was iodinated using a modified chloramine-T method (36) and had a specific activity of approximately 50 Ci/µg. Binding was performed in 1 ml/well of binding buffer (128 mM NaCl-5 mM KCl-5 mM MgSO₄·7H₂O, 0.5 mM CaCl₂-50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5-2.5 µg/ml bovine serum albumin) containing 0.25 ng 125I-TGFβ (approximately 60,000 cpm). Nonspecific binding was determined in the presence of 30 ng of pure porcine unlabeled TGFβ2 and was <25% of the total bound ligand. In this assay, TGFβ1 and β2 are equipotent in displacing 125I-TGFβ2 binding.

For affinity labeling of TGFβ receptors, HS578T and MDA-231 cells were pretreated in 100-mm tissue culture dishes with nonimmune rabbit IgG or 282ab under serum-free conditions. After 24–48 h, the monolayers were washed and incubated in binding buffer while rocking for 4 h at 4°C with 1 ng/ml 125I-TGFβ. After washes with bovine serum albumin-free binding buffer, the bound 125I-TGFβ was cross-linked to cell surface receptors with 50–100 µM disuccinimidyl suberate (Pierce, Rockford, IL) for 15 min (26). The cross-linking reaction was quenched by adding 0.25 M sucrose-10 mM Tris-1 mM EDTA, pH 7.4-0.3 M phenylmethylsulfonyl fluoride, and the cells were harvested by scraping. The cells were pelleted and solubilized with 125 mM NaCl, 10 mM Tris-1 mM EDTA, pH 7.0-1% Triton X-100. Detergent-soluble fractions were electrophoresed in a 5–7% gradient sodium dodecyl sulfate-polyacrylamide gel. Equivalent amounts of total protein determined by the BCA protein assay (Pierce) were loaded in each lane. The gels were dried and exposed to XAR-2 X-ray film at −70°C. In order to quantitate the affinity-labeled TGFβ receptors, laser densitometry of representative autoradiograms was performed.

To determine receptor number and binding affinity, confluent monolayers of cells were incubated in 24-well plates for 4 h at 4°C with 0.25 ng/ml 125I-TGFβ in the absence or presence of cold TGFβ1 (0.25–100 ng/ml). After washings, specifically bound radioactivity was

The following table shows the stimulation of monolayer growth of human breast cancer cells with anti-TGFβ antibodies.

<table>
<thead>
<tr>
<th></th>
<th>HS578T (× 10⁴)</th>
<th>MDA-231 (× 10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>585 ± 19</td>
<td>761 ± 25</td>
</tr>
<tr>
<td>NRG1G</td>
<td>589 ± 14</td>
<td>786 ± 42</td>
</tr>
<tr>
<td>278ab</td>
<td>789 ± 18</td>
<td>899 ± 19</td>
</tr>
<tr>
<td>282ab</td>
<td>791 ± 27</td>
<td>1042 ± 95</td>
</tr>
</tbody>
</table>

Fig. 6. Stimulation of DNA synthesis in quiescent HS578T cells treated with anti-TGFβ antibodies. HS578T cells were maintained in serum-free medium until they become quiescent. At this point, either nonimmune rabbit IgG (○) or 278ab (●) was added to the cells, and [3H]thymidine incorporation was measured 24 h later after a 1-h pulse as described in "Materials and Methods." Each data point, mean of triplicate determinations ± SD (bars).
measured in a gamma counter, and the binding data were subjected to Scatchard analyses.

Cell Proliferation Experiments. For monolayer growth experiments, cells were plated in six-well tissue culture plates in regular growth medium. Twenty-four h later, the medium was changed to serum-free IMEM with or without anti-TGFβ antibodies. Fresh media and antibody were changed every other day. Cells were trypsinized on day 6, and cell number was determined in a model ZF Coulter counter (Coulter Electronics, Inc., Hialeah, FL).

Anchorage-independent growth assays were done in 35-mm Petri dishes with a 1-ml bottom layer of 0.8% agarose (Sea-Plaque; FMC Corp., Rockland, MD)-IMEM-10% calf serum (Hazleton)-10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. A 1-ml top layer containing a single cell suspension of 10^5 cells in 0.8% agarose-IMEM-10% calf serum and different concentrations of anti-TGFβ antibody was added. Dishes were incubated in a 5% CO2 atmosphere at 37°C, and colonies measuring >50 μm were quantitated after 7–14 days using a Bausch & Lomb Omnicron feature analysis stem model II image analyzer. These were the only experiments done in the presence of serum.

For measurement of DNA synthesis, 3–4 × 10^4 cells/well were plated in 24-well plates and changed to serum-free medium 24 h later. Conditioned medium, native TGFβ, or antibodies were added the following day. Eighteen to 24 h later, DNA synthesis was measured by the incorporation of [3H]thymidine [0.5 μCi/ml (82.2 Ci/ mmol; NEN Products, Boston, MA)] into 10% trichloroacetic acid-precipitable material after a 1-h pulse as described (6).

Northern Blot Analysis. Total cellular RNA was extracted by the method of Schwab et al. (37). Oligo(dT)-selected RNA was separated by electrophoresis in a 1.2% agarose-formaldehyde gel (38), and Northern blotting was performed as described previously (39). A 1.0-kilobase Smal insert from a TGFβ cDNA provided by Dr. Rik Derynck (Genentech, San Francisco, CA) was 32P labeled by a random primer extension method (40) and hybridized with the Northern transfers. All membranes were rehybridized with a 32P-labeled 1B15 cRNA probe (41) in order to control for RNA loading and transfer. The intensity of the TGFβ1 transcript was determined by laser densitometry after normalization to the intensity of the constitutively expressed 1B15 transcript.

Acknowledgments
We thank Randy Strough for technical assistance and Leigh Witherspoon for typing the manuscript.

References


