The Antiproliferative Effect of Type β Transforming Growth Factor Occurs at a Level Distal from Receptors for Growth-activating Factors

Transforming growth factor-β (TGFβ) from human platelets blocks the ability of Mv1Lu mink lung epithelial cells to grow in response to serum mitogens, epidermal growth factor (EGF), or insulin. The phenotypic response of Mv1Lu cells to TGFβ is characterized by a flat, very enlarged cell morphology and a markedly increased production and accumulation of extracellular matrix fibronectin. The ability of TGFβ to alter the ligand binding or signal transducing activity of mitogen receptors in Mv1Lu cells has been examined. In contrast to NRK-49F rat fibroblasts, Mv1Lu cells do not respond to TGFβ with a decrease in the affinity or a change in the number of cell surface receptors for EGF. Soluble extracts from Mv1Lu cells contain a protein kinase activity which selectively phosphorylates ribosomal protein S6; this S6 kinase activity is elevated several fold minutes after exposure of cells to mitogens. This kinase activity has been used as the parameter to measure the signaling ability of EGF receptors and insulin receptors in cells treated with TGFβ. We find that TGFβ does not alter the basal level of S6 kinase activity or its elevation by EGF or insulin. In TGFβ-treated cells rendered insensitive to the growth-promoting action of EGF, the parameters of elevation of S6 kinase activity by EGF are similar to those of control, growth-competent cells. The results suggest that TGFβ inhibits cell proliferation by acting at a level distal from the receptors for growth-activating factors.

Transforming growth factor-β (TGFβ), a hormonally active polypeptide found in normal and transformed tissues, is a potent regulator of cell growth and differentiation. TGFβ can induce anchorage-dependent and anchorage-independent growth of fibroblasts in some cases, but it inhibits the growth of normal as well as tumor-derived cells in other cases (Refs. 1-5; for review, see Ref. 6). However, TGFβ is a strong inhibitor of adipogenic and myogenic differentiations under conditions in which it does not alter the proliferation of preadipocytes or myoblasts (7, 8). Many cells whose growth or differentiation is regulated by TGFβ respond to this factor with a marked increase in the production and accumulation of the extracellular matrix proteins fibronectin and collagen (9). Available evidence suggests the possibility of a causal link between accumulation of an abundant extracellular matrix and induction of anchorage-independent growth by TGFβ (9). These actions of TGFβ are presumably mediated by specific cell surface receptors. Three structurally distinct forms of cell surface glycoproteins that exhibit the properties of high affinity receptors for TGFβ have been identified in mammalian and avian cells (10-12). It is not known whether all receptor forms are involved in the mediation of TGFβ actions or whether only one receptor form is a signaling receptor while the others have a different function.

The anchorage-independent proliferation of fibroblasts induced by TGFβ depends on the presence of serum mitogens including EGF, platelet-derived growth factor, and insulin-like growth factors (1, 3, 13, 14). TGFβ also alters the growth response of fibroblasts in monolayer culture, in this case by prolonging the prereplicative period of quiescent cells stimulated with mitogens (15). The ability of TGFβ to modulate the number and affinity of EGF receptors in some cell lines has been proposed as a possible mechanism by which TGFβ modifies cellular responses to EGF (16-18). The implication of this hypothesis is that by altering the ligand binding activity of the receptor, TGFβ would also alter the signal transducing activity, thus changing the quality or intensity of the cellular response to EGF at the primary level. However, this hypothesis has not been substantiated with direct evidence as yet.

The mink lung epithelial cell line Mv1Lu provides the opportunity to determine whether modification of the cellular response to growth factors by TGFβ occurs at a level proximal or distal to the growth factor receptors. The proliferation of Mv1Lu cells is potently inhibited by TGFβ (4, 19). As shown here, TGFβ blocks completely the proliferation of Mv1Lu cells cultured in the presence of EGF, insulin, or serum. If the inhibitory action of TGFβ is at the level of growth factor receptors, it would be expected that the signal transducing activity of the receptors would be markedly impaired after treatment with TGFβ. The parameter that we have selected here to measure the signal transducing activity of mitogen receptors in TGFβ-treated Mv1Lu cells is the elevation of S ribosomal protein S6 kinase activity. As previously shown in fibroblasts (20-22), this kinase activity is sharply elevated within minutes of treatment of Mv1Lu cells with mitogens. The enzyme(s) responsible for this activity has raised considerable interest because it is regarded as one of the primary targets of signals elicited by growth factor receptors. Studies have shown a possible link between increased S6 phosphorylation and the initiation of protein synthesis required for cell proliferation (23). Here we show that elevation of S6 kinase activity by EGF and insulin is not impaired in Mv1Lu cells.
growth-inhibited by TGFβ. These observations indicate that TGFβ exerts its growth inhibitory action at a level distal from the growth factor receptors.

EXPERIMENTAL PROCEDURES AND RESULTS

DISCUSSION

The existence of growth-inhibiting factors along with growth-stimulating factors suggests that normal cell proliferation in the organism is the result of a balance between the actions of both types of factors. Whereas substantial information exists on the events that take place in response to mitogenic factors at the membrane, cytosolic, and nuclear levels, very little is known about the biochemical actions of growth inhibitors, in particular TGFβ. The response of Mv1Lu cells to TGFβ is an extreme example of the growth inhibitory activity of this factor. In contrast to fibroblasts which respond to TGFβ with a limited decrease in the rate of growth in the presence of serum, Mv1Lu epithelial cells respond to TGFβ with a complete arrest of their proliferation. This growth response correlates with a severe change in morphology and abundant deposition of fibronectin in the extracellular matrix. Thus, Mv1Lu cells represent an ideal system to study the molecular basis of growth inhibition by TGFβ.

A possible level at which TGFβ could act to inhibit the mitogenic actions of other growth factors in Mv1Lu cells is the receptors for these growth factors. The EGF receptor is a sensitive target for modulation by other agents that regulate cell proliferation, including platelet-derived growth factor (26–29) and tumor-promoting phorbol esters (30, 31). Both types of agents induce phosphorylation of the EGF receptor molecule at unique sites, the major one of which is threonine 654 (32–34). This reaction is probably catalyzed by protein kinase C (35) and is correlated with changes in the binding and phosphorylating activities of the EGF receptor (34, 36).

Another factor that modulates EGF receptors, at least in NRK-49F fibroblasts, is TGFβ (16–18). TGFβ acts rapidly to reduce the high affinity EGF receptor sites in NRK-49F cells (17, 18), and it can also increase the number of low affinity EGF receptors after prolonged incubation with these cells (16, 18). This biphasic effect of TGFβ on EGF binding has been proposed as the basis for the dual action of TGFβ on the proliferation of NRK-49F cells (18). According to this hypothesis, induction of anchorage-independent proliferation by TGFβ is the presence of EGF or its analogue, TGFα, would be mediated by the increased number of low affinity receptors for EGF/TGFα, whereas partial inhibition of monolayer growth by TGFβ would be mediated by the decrease in high affinity EGF/TGFα-binding sites.

Conclusive proof for this hypothesis is still lacking. However, our results with Mv1Lu cells illustrate a situation in which complete inhibition of the proliferative response to EGF by TGFβ is not linked to a measurable decrease in the binding affinity or capacity for EGF. This finding argues against the possibility that modulation of EGF receptors by TGFβ is a general phenomenon that accounts for the effects of TGFβ on cell proliferation. Alternatively, it is possible that TGFβ inhibits the activity of EGF receptors in Mv1Lu cells in a way which cannot be detected by ligand binding measurements. To address the latter possibility, we have examined activation of S6 kinase as a parameter that should integrate the ligand binding and the signal transducing activities of EGF receptors. The rationale is that a meaningful change in EGF receptors caused by TGFβ should be reflected in their ability to elicit a rapid cellular response such as activation of S6 kinase.

The results demonstrate the presence of a soluble protein kinase activity in Mv1Lu and NRK-49F cells which catalyzes selectively the phosphorylation of exogenous S6 ribosomal protein in vitro. This kinase activity measured in the presence of phosphatase inhibitors is sharply increased in cell extracts prepared minutes after addition of EGF, insulin, or calf serum to the cells. A similar S6 kinase activity has been described in homogenates from mouse 3T3 fibroblasts treated with EGF, insulin, or calf serum (20–22) and Rous sarcoma virus-transformed chick embryo fibroblasts (37). The enzyme(s) responsible for this activity in mouse 3T3-L1 fibroblasts has been shown to be different from any other known protein kinase (22) and is thought to be responsible for the extensive phosphorylation of ribosomal protein S6 that occurs in vivo minutes after treatment of cells with mitogenic agents. Determination of the phosphorylation of S6 in both sets of activation in Mv1Lu cells shows that it can be induced by picomolar concentrations of EGF and is saturated at 1 nM EGF (Figs. 5 and 6). Interestingly, the kinetics of elevation and decrease of S6 kinase activity after addition of EGF to Mv1Lu cells in the present studies are very similar to the kinetics in fibroblasts previously reported (21). Previous studies have shown that the transient nature of S6 kinase activation by EGF correlates with the down-regulation of cell surface EGF receptors that occurs shortly after treatment of cells with EGF (21).

The results reported here show that S6 kinase activity is not regulated directly by TGFβ in Mv1Lu cells or NRK-49F cells. Addition of TGFβ to the cells did not alter the measurable activity of the kinase under any of the conditions of time (2 min to 2 days) or TGFβ concentrations (3 pm to 1 nM) tested (not shown). Treatment of cells with TGFβ shortly before the addition of EGF or insulin does not alter the effect of these factors on S6 kinase activity in either cell line, even though a decrease in EGF receptor binding activity may occur in NRK-49F cells under these experimental conditions (17). More important, Mv1Lu cells that had been rendered insensitive to the mitogenic action of EGF, insulin, or calf serum by exposure for 2 days to TGFβ exhibited a normal response of S6 kinase activity to EGF when compared with control growth-competent cells. The extent of elevation of S6 kinase activity, the kinetics of this effect, and the sensitivity to various concentrations of EGF were similar in both sets of cells. These findings indicate that the ability of EGF receptors to signal for S6 kinase activation, and possibly other cellular responses, is not impaired in the growth-inhibited state. The results do not exclude the possibility that TGFβ induces a molecular alteration on the EGF receptor which is unrelated to ligand binding or S6 kinase activation but blocks the mitogenic action of EGF. Although this possibility cannot be ruled out, it is unlikely because TGFβ also inhibits the growth response to insulin and serum mitogens which act through receptors and mechanisms distinct from, albeit related to, those of EGF. These observations then lead to the conclusion that inhibition of the proliferative response by TGFβ occurs at a level distal from the receptors for growth-activating factors.
Supplement to
The Antiproliferative Effect of Type I Transforming Growth Factor-α (TGF-α) on Human Bladder Cancer Cell Cultures
by
Setty Lee and Joan Massague

EXPERIMENTAL PROTOCOLS

Cell Culture

Stock cultures of MiI-12 and U-373MG cells (SEL, American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin-streptomycin. Cells were plated at a density of 2 x 10⁴ cells/cm². After 24 h, cell culture supernatants were replaced with media containing 0.1% of either TGF-α or control supernatant. After 48 h, cell culture supernatants were harvested and assayed for growth inhibitory activity.

Proliferation Assays

Cells were seeded in 60 mm tissue culture dishes such that they would achieve the desired degree of monolayer density (about 1 x 10⁴ cells/cm²) at the time of treatment with anti-growth factors. Cells were then cultured in 5% CO₂ at 37°C for various time periods. Cells were then counted by trypsinization and trypan blue exclusion.

Protein kinase Activity Assays

Cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, and 100 μg/ml aprotinin) and solubilized in 5 M urea. The extracts were then subjected to electrophoresis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were then stained with Coomassie Blue R-250 and dried before autoradiography.

Protein kinase activity was assayed by measuring the incorporation of ¹²⁵I-iodo-mannose into the cell lysates. Cell lysates were incubated with [¹²⁵I]-iodo-mannose and [³H]-proline in the presence of [¹²⁵I]-iodo-mannose and were separated by SDS-PAGE. The gels were then exposed to X-ray film to detect the incorporation of radioactive material. The results were analyzed by densitometry.

RESULTS

Growth Inhibition by TGFβ

The sources of homogeneous preparations of TGFβ, if and insulin were from American Type Culture Collection.

Growth Factors

The role of TGFβ in the growth inhibition of human bladder cancer cells was tested by incubating the cells with TGFβ in the presence or absence of insulin or serum. The results showed that TGFβ inhibited the growth of the cells in a dose-dependent manner.

Figure 1. Effect of TGFβ on the Proliferation and Morphology of MiI-12 Cells

Growth inhibition of MiI-12 cells by TGFβ was observed in a dose-dependent manner. The cells showed a decrease in cell number and an increase in cell size. The inhibition of cell proliferation was accompanied by a decrease in the rate of DNA synthesis, as measured by the incorporation of thymidine into DNA.

Discussion

The results of this study indicate that TGFβ has an inhibitory effect on the growth of human bladder cancer cells. The mechanism of this inhibition is not fully understood, but it is possible that TGFβ may be acting through the regulation of cell cycle progression.

REFERENCES


The growth response of NRK-49F rat kidney fibroblasts after treatment with TGFβ. The lack of Rap-dependent effect of TGFβ on the proliferation of WIL2 cells was illustrated (Fig. 3). This effect of TGFβ in isolated mitogens. The kinetic of activation of the ability of mitogen receptors to activate protein kinase is shown. The maximal three-fold elevation of TGFβ activity was observed after 10 min of exposure to EGF, the kinase activity started to decline after 60 min of exposure to EGF, and was restored again by the subsequent addition of TGFβ. The stimulation of the kinase activity by TGFβ receptor appears to be unique in WIL2 cells growth-inhibited by TGFβ.

Figure 3. Effect of TGFβ on Growth Inhibition by TGFβ, EGF, or Insulin in NRK-49F Cells.

- Cells were seeded in medium containing 0.15% BSA, and treated with 0.6 μM TGFβ. After 4 h, cells received 0.6 μM TGFβ and Insulin. The cells were counted immediately after these incubations. Results are shown.

Figure 4. Effect of Growth Factors on EGF Kinase Activity in WIL2 Cells and NRK-49F Cells.

- Cells were maintained for 12 h in low serum medium. Some cultures then received 0.3 μM TGFβ. One h later, additions of 0.3 μM EGF, 1 μM Insulin, 0.6 μM TGFβ, or nothing were made. After 20 min, whole cell extracts were obtained and analyzed for EGF phosphorylation. The specific activity of [3H]-EGF phosphorylation extracts was determined using an antibody to phosphotyrosine and autoradiography. The data in the individual mitogens, EGF, and Insulin, show that TGFβ action correlated with a decrease in TGFβ treated cells (2.4 × 10^6 cpm/10^6 cells).

Table 1. Effect of TGFβ on the Kinase Activity in WIL2 Cells and NRK-49F Cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TGFβ Control</th>
<th>TGFβ Insulin</th>
<th>TGFβ EGF</th>
</tr>
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<tbody>
<tr>
<td>WIL2</td>
<td>24.7 ± 1.5</td>
<td>19.6 ± 0.7</td>
<td>17.5 ± 0.4</td>
</tr>
<tr>
<td>NRK-49F</td>
<td>36.2 ± 2.3</td>
<td>28.3 ± 1.1</td>
<td>25.1 ± 0.8</td>
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Figure 5. Parameters of EGF Kinase Activation by TGFβ in WIL2 Cells.

- Cells were placed in low serum medium for 12 h. Some cultures then received 0.6 μM TGFβ. After 4 h, cells received 0.6 μM TGFβ and Insulin. In those treated with 0.6 μM TGFβ, the ability of mitogen receptors to activate protein kinase was measured (Fig. 3). The data in the individual mitogens, EGF, and Insulin, show that TGFβ action correlated with a decrease in TGFβ treated cells (2.4 × 10^6 cpm/10^6 cells).

Figure 6. Activation of EGF Kinase in Control and Growth-Inhibited Cells.

- Cells were plated in low serum medium (a) or with 0.6 μM TGFβ (b) for 2 days. Cells were then incubated with 0.6 μM TGFβ for 10 min in medium with 0.15% BSA, and counted immediately after these incubations. Results are shown.