Reversible Inhibition of Normal Human Prokeratinocyte Proliferation by Type β Transforming Growth Factor-Growth Inhibitor in Serum-free Medium

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ABSTRACT

Type β transforming growth factor-growth inhibitor (TGFβ/GI) causes normal human prokeratinocytes to arrest growth predominantly in the G1 phase of the cell cycle within 48 h after log phase cultures are exposed to the factor in serum-free medium. The growth arrest induced by TGFβ/GI is reversible because the cells from treated cultures can be replated into fresh medium and grown into large colonies. Normal prokeratinocytes are demonstrated to secrete TGFβ/GI-like molecules into the culture medium and to have specific cell surface receptors for this molecule. In contrast, a human squamous cell carcinoma, SCC-25, does not arrest growth when exposed to TGFβ/GI. These cells, unlike the normal prokeratinocytes, do not exhibit detectable cell surface receptors for the factor.

INTRODUCTION

TGFs may be defined as polypeptides that have the ability to cause normally anchorage-dependent mesenchymal cells to form colonies in semisolid agar (see Refs. 1 and 2 for reviews). Two separate TGFs have been well characterized. TGFα is a small, single-chain, acid- and heat-stable molecule that has sequence homology to EGF, binds to the EGF receptor, and stimulates cell proliferation (3—5). TGFβ is a Mr 25,000 acid-stable molecule composed of two apparently identical polypeptide chains that has unique cell surface receptors on a variety of cell types (6—9). It stimulates AKR-2B cells, rat NRK cells, and normal human fibroblasts to form colonies in soft agar in medium that contains serum (1, 6, 10). TGFβ also stimulates DNA synthesis in serum-free monolayer cultures of AKR-2B cells with unique kinetics (11). However, under certain circumstances TGFβ can also inhibit the growth of some cells and it has been shown that TGFβ is similar if not identical to a growth inhibitor isolated from medium conditioned by BSC-1 monkey kidney cells (12). This growth inhibitor causes the G1 arrest of low density cultures of BSC-1 cells and other cell lines (13—16). We have, therefore, used the term TGFβ/GI to refer to this molecule(s). TGFβ/GI-like molecules have been isolated from a variety of sources including transformed and nontransformed cells in culture (2, 8, 16, 17), neoplastic tissues (18), and nonneoplastic tissues including bovine kidneys and human platelets (6, 10, 19—21).

In this report we present data demonstrating that TGFβ/GI inhibits the clonal growth of normal human epithelial cells in a defined medium in a dose-dependent fashion. In addition, TGFβ/GI causes a reversible, cell cycle-dependent growth arrest of normal human prokeratinocytes when added to cultures growing logarithmically. In contrast, the data show that the clonal growth of a squamous cell carcinoma (SCC-25) cultured under similar conditions was not affected by the addition of TGFβ/GI and that high density logarithmically growing cultures failed to display cell cycle-dependent growth inhibition. Scatchard analysis of 125I-labeled TGFβ/GI binding to cells that are inhibited or not inhibited revealed the absence of specific binding of TGFβ/GI to the noninhibited carcinoma cells.

MATERIALS AND METHODS

Cell Culture. Human foreskin prokeratinocytes were isolated from newborn foreskin by the trypsin float technique and stock cultures were grown in serum-free medium as described previously (22). The basal growth medium used for the experiments reported here was medium MCDB 153 (1 x 10^-5 M Ca^2+)(23). For stock cultures the basal medium was supplemented with EGF (10 ng/ml), insulin (5 μg/ml), hydrocortisone (1.4 x 10^-5 M), ethanolamine (1 x 10^-5 M), phosphoethanolamine (1 x 10^-5 M), and bovine pituitary extract, BPE (140 μg/ml). The supplemented medium is referred to in the text as "complete medium." For experiments, the complete medium was used with or without BPE as described in the text and the legends to the figures. The human squamous cell carcinoma strain, SCC-25, was purchased from the American Type Culture Collection (Rockville, MD) and was routinely grown in complete medium with or without supplementation with 0.5% fetal calf serum.

Growth Factors. EGF was purified from male mouse submaxillary glands by the method of Savage and Cohen (24). TGFβ/GI was purified from human platelets by the method of Assion et al. (6) with the addition of a final purification step using a Beckman C18 high performance liquid chromatography column eluted with a 40—60% acetonitrile gradient as described previously (9). TGFβ/GI was demonstrated to be pure by sodium dodecyl sulfate-polyacrylamide gel electrophoreses under both reducing and nonreducing conditions as described previously (1). Insulin (bovine), hydrocortisone, and other media components were purchased from Sigma Chemical Co. (St. Louis, MO).

Clonal Growth Assays. Clonal growth assays were performed in 60-mm dishes (Corning) as described previously (22). After the growth period the cells were fixed in 10% formalin in buffered saline solution and stained with crystal violet, and representative plates were photographed.

Cell Cycle Analysis. To determine the cell cycle distribution of normal prokeratinocytes and SCC-25 cells, the cells were removed from the culture surface with trypsin, fixed, stained with mithramycin, and analyzed using a FACs IV cytometer as described previously (22).

TGFβ/GI Radioreceptor Assays. Purified TGFβ/GI was iodinated using Bolton and Hunter reagent as described previously (9) to a specific activity of 100,000 dpm/ng. The binding assays were performed essentially as described for other cell types (9) except that the cells were removed from the culture surface using a solution of 1% Triton X-100 (7). Nonspecific binding was determined in the presence of unlabeled TGFβ/GI (1 μg/ml). For the binding assays, the cells were plated in 6-well dishes at a density of 1 x 10^5 cells/well (1.04 x 10^6 cells/cm²). Binding assays were performed 2—4 days after the cells were plated. To determine whether the conditioned medium from prokeratinocytes and SCC-25 cells contained TGFβ/GI-like activity, varying concentrations of conditioned medium were tested for their ability to inhibit the binding of 125I-labeled TGFβ/GI (0.2 ng/ml) to AKR-2B (clone 84A) cells after acidification of the medium to pH 1.5 and reneutralization to pH 7.0 (25).

Conditioned Medium. To obtain conditioned medium from high...
density cultures of prokeratinocytes and SCC-25 cells, first passage prokeratinocytes or 12th passage SCC-25 cells were plated in complete medium MCDB 153 at $5 \times 10^5$ cells/cm$^2$. Two days after plating the medium was changed to complete medium without BPE that was supplemented with a mixture of amino acids at concentrations higher than those in the basal medium (26). Thereafter the medium was changed every 2 days. This high amino acid medium prevents the cells from arresting at low density due to nutrient depletion as described previously (22) and allows the normal prokeratinocytes and the SCC-25 cells to grow to confluency (2–3 $\times 10^5$ cells/cm$^2$). When the cells became confluent (6–8 days) the medium was changed again and conditioned medium was collected 48 h later.

RESULTS

Effect of TGF/β/IGI on the Clonal Growth of Prokeratinocytes and SCC-25 Cells. We have noted previously that the addition of TGF/β/IGI to cultures of proliferating human prokeratinocytes caused a marked reduction in $[^{3}H]$thymidine labeling within 24–48 h (1). To determine whether TGF/β/IGI has a similar effect on the long-term growth of these cells at low density, we performed clonal growth assays with these cells in a defined serum-free medium. In addition, we compared the effect of TGF/β/IGI on normal human prokeratinocytes to its effect on a human squamous carcinoma cell line, SCC-25, that was grown continuously in our laboratory in the same serum-free medium. While the growth of SCC-25 cells in serum-free medium in stock cultures (seeding density, 1–5 $\times 10^3$ cells/cm$^2$) was as good as the growth obtained with the normal prokeratinocytes, clonal growth of SCC-25 cells in the serum-free medium was reduced in comparison to the growth of the normal cells. However, excellent growth of SCC-25 cells at clonal densities can be achieved if the medium is additionally supplemented with a low concentration of serum (0.5%).

The clonal growth of normal prokeratinocytes was inhibited by the addition of TGF/β/IGI in a dose-dependent fashion (Fig. 1A). Inhibition of growth was detectable when as little TGF/β/IGI as 1.0 ng/ml was added to the medium and clonal growth was drastically reduced at 10 ng/ml. At TGF/β/IGI concentrations above 10 ng/ml essentially no colonies were observed, although microscopic examination of the plates revealed that the cells were still attached at the end of the assay period. When SCC-25 cells were assayed under similar conditions, however, there was no effect of TGF/β/IGI on the clonal growth of these cells at any of the concentrations tested (Fig. 1B). In other experiments on SCC-25 cells where the low concentration of serum used in the studies presented in Fig. 1B was omitted, the size of the colonies obtained was slightly reduced, but TGF/β/IGI had no effect on the growth achieved when tested over the same concentration range (data not shown).

TGF/β/IGI Treatment of Prokeratinocytes Causes a Reversible G$_1$ Arrest. To determine whether the inhibition of clonal growth in prokeratinocytes was due to a specific cell cycle arrest of these cells, prokeratinocytes were grown in complete medium MCDB 153 and the effect of TGF/β/IGI treatment was examined by flow microfluorimetry. We have shown previously that human prokeratinocytes can be grown logarithmically in complete medium MCDB 153 with a population-doubling time of approximately 24 h (22). Table 1 summarizes the data obtained from TGF/β/IGI-treated and untreated cultures. A comparable proportion of cells apparently arrested in the G$_{2}$-M phase has been noted in previous studies in which arrest was induced by other agents (22, 26). After 48 h of TGF/β/IGI treatment, few cells remain in S phase compared to control cultures whereas a large increase in the number of cells with G$_1$ DNA content is apparent; there is little change in the G$_{2}$-M compartment. Cell counts performed on identical cultures demonstrate that TGF/β/IGI inhibited growth (Table 1). In contrast, when SCC-25 cells were treated with TGF/β/IGI under identical conditions there was no effect on the cell cycle distribution of the cells after 48 h of treatment and there was no reduction in cell growth when compared to controls (Table 1).

To determine whether the inhibition of growth of the normal prokeratinocytes was reversible, we arrested logarithmically growing cultures by treating them with TGF/β/IGI (30 ng/ml) for 48 h and then removed them from the culture flask and plated them into a clonal growth assay. Cells previously treated for 48 h with TGF/β/IGI formed colonies with essentially the same efficiency as untreated control cultures (Fig. 2). These results demonstrate that the arrest state induced by TGF/β/IGI in prokeratinocytes is reversible.

TGF/β/IGI Binding to Prokeratinocytes and SCC-25 Cells. To determine whether the difference in the responsiveness of prokeratinocytes and SCC-25 cells to the inhibitory effects of TGF/β/IGI is due to different levels of binding of TGF/β/IGI to these cells, we have examined the binding affinity and the number of cell surface TGF/β/IGI receptors on prokeratinocytes and SCC-25 cells using a $^{125}$I-labeled TGF/β/IGI binding assay described previously (9). Scatchard analysis of the binding of $^{125}$I-TGF/β/IGI to prokeratinocytes at room temperature re-

![Fig. 1. The effects of TGF/β/IGI on the clonal growth of human prokeratinocytes and squamous carcinoma cells. In A, human prokeratinocytes were plated at 500 cells/60-mm dish in complete medium without BPE in the presence of the indicated concentration of TGF/β/IGI. In B, SCC-25 cells were plated at 500 cells/60-mm dish in complete medium without BPE but containing 0.5% fetal bovine serum and the indicated concentration of TGF/β/IGI. The cultures were incubated for 10 days without a medium change and the cells were fixed and stained as described in "Materials and Methods."](image-url)
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We have demonstrated in this report that TGFβ/GI inhibits the growth of normal human prokeratinocytes that are isolated from newborn human foreskin and grown in defined medium that does not contain serum. We have also shown that TGFβ/GI inhibits clonal growth of these cells and that TGFβ/GI-induced growth inhibition is due to the reversible accumulation of these cells in the G1 phase of the cell cycle. The prokeratinocytes have specific cell surface receptors for TGFβ/GI while the human squamous cell carcinoma cell line, SCC-25, has no detectable receptors; the growth of these cells is unaffected by the addition of TGFβ/GI.

Inhibition of the growth of normal human epithelial cells by serum and factors released from platelets has been reported previously for prokeratinocytes (22, 23, 27), mammary epithelial cells (28), and bronchial epithelial cells (29, 30) grown in serum-free medium with supplementation similar to that used in the present studies. Thus, it seems likely that at least one of the factors involved in the inhibition of growth by serum or platelet lysates noted in these previous studies was due to TGFβ/GI which is released from platelets (10). Other factors may also be involved in the control of cell cycle progression and differentiation in human prokeratinocytes, however, because it has been shown that razoxane and certain lymphokines can also inhibit the proliferation of human prokeratinocytes (26).

Binding studies using 125I-TGFβ/GI have shown that TGFβ/GI receptors are present on a wide variety of cells including mesenchymal and epithelial cells (9). While other growth factors (e.g., EGF) have receptors on different cell types, only TGFβ/GI has the property of being stimulatory for some cells (mostly mesenchymal) and inhibitory for other (mostly epithelial) cells (1). It is not clear at this time whether the difference in the responsiveness of different cell types is due to the cells having different receptors for TGFβ/GI or the same receptor mechanism causing stimulation in one setting and inhibition in another. The affinity for TGFβ/GI by its receptor on mouse AKR-2B (clone 84A) cells and rat NRK (clone 49F) cells is higher (approximately 40 pm) (7, 9) than the affinity of the TGFβ/GI receptor on prokeratinocytes. This may reflect the fact that the 84A and 49F mesenchymal cell clones were selected for their hypersensitivity to TGFβ/GI stimulation of growth in soft agar. Experiments with human fibroblasts isolated from foreskin indicate that the Kd of TGFβ/GI for its receptor on these cells is similar to that of prokeratinocytes.

The absence of TGFβ/GI binding to SCC-25 cells could be due to continual occupancy and/or down regulation of the receptor by endogenous TGFβ/GI. However, preliminary studies indicate that the SCC-25 cells produce no more TGFβ/GI than the normal prokeratinocytes and no TGFβ/GI receptor-competit activity is detectable in unprocessed conditioned medium from
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SCC-25 cells. Alternatively, the cells may produce an aberrant receptor or no receptor.

The fact that the growth of the human carcinoma cell line, SCC-25, is not inhibited by TGFβ/G1 while normal human prokeratinocytes become arrested in the G1 phase of the cell cycle raises the question of what normal role, if any, TGFβ/G1 has in the regulation of normal cell growth in vivo and whether alterations in these mechanisms may play a role in the development of carcinomas. We have postulated that if TGFβ/G1 plays a role in the regulation of growth of normal epithelial cells (via a negative control mechanism) then a change in this mechanism could lead to altered growth control in carcinomas (1, 11). The data presented here demonstrate that normal human prokeratinocytes respond to TGFβ/G1-like molecules whereas SCC-25 human squamous carcinoma cells do not. These findings support the hypothesis that this molecule may play a role in normal growth regulation in these cells in a manner similar to that proposed by Holley et al. (13) for BSC-1 cells. Like BSC-1 cells, prokeratinocytes release TGFβ/G1 into the medium (see Table 2); however, most appears to be in an inactive form because no receptor-competing activity is detectable without acid treatment in agreement with the results of Lawrence et al. (25) with fibroblastic cells. In addition, the inability of the SCC-25 cells used in this study to respond to the growth-inhibitory effects of TGFβ/G1 suggests that loss of responsiveness to TGFβ/G1 could lead to aberrant growth or that aberrant growth leads to loss of responsiveness to TGFβ/G1.

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REFERENCES


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