**ABSTRACT**

The effects of exogenously added transforming growth factor (TGFα and TGFβ) on the growth of BALB/MK cells were examined. TGFα supplanted the epidermal growth factor (EGF) requirement in these cells. In contrast, TGFβ reversibly inhibited the growth of BALB/MK cells by abrogating the stimulatory actions of EGF or TGFα. The inhibitory effects of TGFβ appeared to be mediated by events distal to EGF ligand-receptor interactions. Growth inhibition of BALB/MK cells by TGFβ did not result in the induction of differentiation. This finding is different from the growth inhibition of these cells induced by elevated calcium levels (1.5 mM) which was tightly coupled to terminal differentiation. The BALB/MK cells were found to express TGFα mRNA, as well as TGFβ mRNA and protein. In addition, TGFα, as well as EGF, enhanced TGFα gene expression. These studies suggest a role for endogenous TGFα in regulating BALB/MK proliferation. TGFα provides a positive growth signal, while TGFβ is a potent inhibitor of growth even in the presence of such positive modulators as TGFα and EGF.

**INTRODUCTION**

While TGFs have been operationally defined as polypeptides that stimulate anchorage-dependent mesenchymal cells to form colonies in soft agar, it is becoming apparent that these molecules modulate normal cellular growth and proliferation (1-4). To date, two classes of TGFs have been extensively characterized, TGFα and TGFβ.

TGFα is a single-chain polypeptide with a molecular weight of 5619 (5). It shares with EGF structural homology and a common receptor, binding to which appears to mediate the actions of both molecules (4, 5). TGFα and EGF exhibit a similar, although not identical, spectrum of biological activity (4, 6). TGFα was first identified in medium conditioned by sarcoma virus-transformed cells and called sarcoma growth factor (7); it was later found in embryonic and placental tissue extracts (8, 9). TGFα has been considered as an embryonic form of EGF that is inappropriately expressed in neoplasia. Recently, however, TGFα expression has been observed in nontransformed cells, in vitro and in vivo (10, 11). EGF has been shown to stimulate a number of epithelial cell types (12, 13).

TGFβ is a homodimer with a molecular weight of 25,000 that differs from TGFα in its molecular structure (13) and biological activity (2). TGFβ is produced by both normal and neoplastic cells (2). Secreted in an inactive form, TGFβ has been shown to be activated by nonphysiological extremes of pH

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**MATERIALS AND METHODS**

Materials. Porcine TGFβ, form 1, was provided by R & D Systems, Inc. (Minneapolis, MN). Synthetic rat TGFα was obtained from Peninsula Laboratories (Belmont, CA).

Cell Culture. BALB/MK cells were maintained in minimum essential medium containing 0.05 mM calcium supplemented with 8% dialyzed fetal bovine serum (GIBCO Laboratories, Grand Island, NY) and 4 ng/ml EGF (Collaborative Research, Bedford, MA).

DNA Synthesis Measurements. Growth factor effects on DNA synthesis were evaluated by [3H]thymidine incorporation and autoradiography. For [3H]thymidine incorporation assays, BALB/MK cells were plated in 24-well dishes in maintenance medium (4 x 10^4 cells/well), and experiments were performed when the dishes reached 60-80% confluency. For experiments utilizing quiescent cells, the cells were changed into medium lacking EGF when they reached 60-80% confluency. The cells remained in EGF-free medium for 48 h prior to the onset of the experiment. Cells were labeled with [3H]thymidine (50 to 80 Ci/mmol; New England Nuclear, Boston, MA) at a concentration of 4 μCi/ml for the indicated times. After the pulse, the relative amount

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2 This work was supported in part by United States Public Health Services Grants CA 42572, CA 42749, and CA 42950 (H. L. M.), and CA 36936 (B. W.) awarded by the National Cancer Institute, Department of Health and Human Services.
3 The abbreviations used are: TGF, transforming growth factor; EGF, epidermal growth factor; PBS, phosphate-buffered saline.
5 Stuart H. Yuspa, personal communication.
of radioactivity incorporated into trichloroacetic acid-insoluble material was determined by scintillation counting in an aqueous fluor (American/Searle Corp., Arlington Heights, IL).

 Autoradiography was performed as previously described (22). Briefly, the cells were plated in 35-mm dishes at a concentration of 6 \times 10^4 cells/dish. When cells reached 60–80% confluence, they were changed into medium lacking EGF. After 48 h, one group of dishes received fresh medium lacking EGF, one group received medium containing EGF, and one group received medium containing EGF plus TGFβ (10 ng/ml). Dishes from each group were labeled with 8 \muCi/ml \[^{3}H\] thymidine for 0–30 and 30–60 h. At 30 h, some dishes in the last group were given fresh medium with EGF but without TGFβ. These dishes were then labeled for 30–60 and 60–90 h. At least 250 cells were counted per dish.

 Macromolecular Synthesis Determinations. BALB/MK cells (4 \times 10^4 cells/well) were plated in 24-well dishes. At 60–80% confluence, the cells were treated with various concentrations of TGFβ. After 5 h at 37°C, the cells were labeled with 10 \muCi of \[^{3}H\] thymidine (5.6-\[^{3}H\]-thymidine, 47.1 Ci/mmol, New England Nuclear) or \[^{35}S\]methionine (1.5 Ci/mmol, New England Nuclear) for 1 h. At this time, RNA and protein acid precipitable counts were determined as described for DNA synthesis measurements.

 EGF Binding Studies. BALB/MK cells were plated in six-well dishes (1 \times 10^5 cells/well) in maintenance medium. When the cells were 60–80% confluent, the dishes were changed to medium lacking EGF for 12–24 h. At this time, cells were treated with TGFβ (10 ng/ml) for the times indicated. The dishes were washed three times with PBS, and 0.185 ng of \[^{125}I\]EGF (specific activity, 8.1 \times 10^7 cpm/\mug; labeled by the chloramine T reaction) was added to each well in binding buffer (PBS, 0.1% BSA, 5 mM MgCl₂, pH 7.4). After 1 h incubation at 4°C, the dishes were washed three times with PBS. Cells were solubilized in 1 ml of 0.2 N NaOH and removed for gamma counting. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled EGF and never exceeded 15% of specific counts in any determination.

 For internalization assays, the cells in medium lacking EGF were treated with TGFβ (10 ng/ml) for 12 h. At this time, \[^{125}I\]EGF was added to the cultures and they were incubated for 30 min or 1 h at 37°C. At the specified time, the dishes were placed at 4°C and washed three times with cold PBS. The monolayers were washed two times with 1 ml of 0.2 M acetic acid, 0.5 M NaCl, pH 2.5 for 6 min. These washes were collected as surface bound ligand. The cells were then solubilized with 0.2 N NaOH for determination of internalized ligand. Both samples were counted in a gamma counter.

 Measurement of TGFβ Activity in BALB/MK Conditioned Medium. BALB/MK cells were grown to 80% confluence in 175-cm² flasks (Falcon, Oxnard, CA). After three washes in buffered saline solution A (23), the medium was changed to serum-free minimum essential medium. The first 24-h collection was discarded, and the next 48-h collection was collected and centrifuged at 5000 \times g for 30 min. Cell numbers were determined by hemocytometer counts. An aliquot of the cultures and they were incubated for 30 min or 1 h at 37°C. At the specified time, the dishes were placed at 4°C and washed three times with cold PBS. The monolayers were washed two times with 1 ml of 0.2 M acetic acid, 0.5 M NaCl, pH 2.5 for 6 min. These washes were collected as surface bound ligand. The cells were then solubilized with 0.2 N NaOH for determination of internalized ligand. Both samples were counted in a gamma counter.

 RESULTS

 Effects of TGFα and TGFβ on Proliferation of BALB/MK Cells. Since TGFα and EGF are structurally related and interact with the same receptor, we initially examined whether TGFα could replace the EGF requirement for growth and proliferation of BALB/MK cells. Cells were grown arrested by deletion of EGF from the growth medium for 48 h and equimolar amounts of EGF and TGFα tested for their ability to stimulate DNA synthesis. Fig. 1 demonstrates that TGFα and EGF are very comparable in their mitogenicity for BALB/MK cells.

 TGFβ has been shown to inhibit the growth in culture of all normal epithelial cells thus far studied (17, 19), including human and mouse keratinocytes (2, 16, 30). TGFβ also inhibited proliferation of BALB/MK cells. Subconfluent, rapidly growing BALB/MK cells show half maximal inhibition of DNA synthesis at 2 ng/ml of TGFβ as measured by a pulse of \[^{3}H\] thymidine 24 h following treatment with TGFβ (Fig. 2). Inhibition of clonal growth of these cells was also demonstrated with a similar dose response (data not shown). To test the
Mechanism(s) of TGFβ Inhibition of EGF Stimulation. To initially examine the mechanism(s) by which TGFβ inhibits growth factor action, we determined the effect of TGFβ on total RNA and protein synthesis as reflected by labeled precursor incorporation. While TGFβ is a potent inhibitor of DNA synthesis, it had little or no effect on incorporation of [3H]uridine or [35S]methionine at 6 h following its addition to rapidly growing cells (Fig. 2). Thus, the data suggest that the mechanism of inhibition of BALB/MK cell proliferation by TGFβ is probably not through general inhibition of RNA or protein synthesis.

Since TGFβ inhibits EGF stimulation of BALB/MK cells, it is possible that TGFβ blocks BALB/MK proliferation by altering binding of EGF to its receptor. Fig. 5 demonstrates that treatment with TGFβ does not significantly affect [125I]EGF binding or internalization in BALB/MK cells. These results are in contrast to the divergent findings by two groups in NRK fibroblasts (32, 33) but consistent with findings in nontransformed (34) and transformed (32) epithelial cells. Thus TGFβ growth inhibition of BALB/MK cells appears to be mediated by events distal to EGF ligand receptor binding or events independent of the EGF pathway.

TGFβ has been shown to promote terminal squamous differentiation in human bronchial epithelial cells (18). When exposed to extracellular calcium levels greater than 1.0 mM, BALB/MK cells will undergo differentiation (20). Terminal differentiation in these cells is characterized by a dramatic change in morphology as well as the expression of many new proteins which have been associated with epidermal differentiation.
expression of all three proteins was detected (Fig. 6, d-f). However, when BALB/MK cells were treated with TGFβ for 24 h, no marker protein expression was seen (Fig. 6, g-i). Thus, the mechanism by which TGFβ inhibits the growth of BALB/MK cells does not appear to be mediated through an induction of terminal differentiation.

Production of TGFα and TGFβ by BALB/MK Cells. It has recently been demonstrated that normal human keratinocytes express TGFα in vitro and in vivo and that EGF and TGFα enhance TGFα expression in these cells maintained in a serum-free system (11). Fig. 7 shows that the same phenomenon occurs in BALB/MK cells. Polyadenylated RNA was isolated from cultures of previously quiescent BALB/MK cells that had been restimulated for 4 h with 10 ng/ml of EGF or TGFα. These samples were analyzed by Northern hybridization for the presence of TGFα mRNA. In comparison to RNA isolated from quiescent cultures of BALB/MK cells, the EGF or TGFα-treated cultures showed an equivalent enhancement of a 4.5-4.8-kilobase transcript which is consistent in size with TGFα mRNA from SW620 cells (Fig. 7) which are known to express TGFα (41, 42). TGFα protein levels were not measured due to the unavailability of antibodies specific to mouse TGFα.

Human foreskin keratinocytes in secondary culture under serum-free conditions have also been shown to produce TGFβ as determined by radio-receptor assay (16). TGFβ expression by BALB/MK cells was determined both by Northern blot analysis of RNA and radio-receptor assay of serum-free conditioned medium. Polyadenylated RNA isolated from rapidly growing cultures contained TGFβ mRNA (Fig. 8A). In addition, acid-activated, serum-free medium conditioned by BALB/MK cells contains TGFβ activity at the level of 100 pg/10⁶ cells; however, medium not pretreated with acid showed no receptor competing activity (Fig. 8B).

DISCUSSION

Understanding the mechanisms that control normal epithelial cell growth and differentiation is essential for understanding the potential defects in growth regulation that occur in malignant cells. BALB/MK cells provide an epithelial model system for the evaluation of such regulatory pathways. These cells are dependent on EGF for their continued proliferation, and the present studies demonstrate that equimolar concentrations of TGFα can supplant this growth requirement for EGF. TGFβ is demonstrated to reversibly inhibit DNA synthesis and subsequent cell division of BALB/MK cells. These inhibitory responses of BALB/MK cells to TGFβ are in agreement with results in other epithelial systems (15–19). Our studies demonstrate that TGFβ inhibits both exponentially growing and EGF-stimulated quiescent cultures of murine keratinocytes, and these effects are specific to DNA synthetic events with minimal effect on synthesis of other macromolecules. To date, fibroblastic cells have been used most extensively to study mechanisms of TGFβ effects. Yet, fibroblasts have complex responses to TGFβ, and the growth stimulation of fibroblasts involves a delay in the EGF plus insulin-induced peak of DNA synthesis (31). Also, TGFβ is probably an indirect mitogen in AKR-2B cells by induction of c-sis transcription with subsequent production of platelet-derived growth factor (43). In contrast to this complicated process in fibroblasts, the kinetics of TGFβ inhibition of EGF mitogenicity in BALB/MK cells are more straightforward. TGFβ appears to suppress the peak of EGF-induced DNA synthesis at 24 h.

BALB/MK cells are also sensitive to growth inhibitory sig-
Fig. 6. Expression of epidermal differentiation markers in calcium and TGF\(\beta\)-treated BALB/MK cells. To determine the differentiation phenotype of BALB/MK cells under various conditions, three markers of epidermal differentiation were examined by immunofluorescent techniques. Cells were grown on coverslips and treated with either calcium chloride (1.0 mM) TGF\(\beta\) (10 ng/ml) for 24 h. Coverslips were then stained immediately for pemphigus vulgaris antigen or after methanol:acetone fixation for desmoplakin or filaggrin. Photomicrographs of representative areas of each coverslip are presented. Cells which are positive for the pemphigus vulgaris antigen display outer membrane fluorescence. Desmoplakin, a component of desmosomes, is found at the cell junctions. Filaggrin, which is involved in keratin filament aggregation, is present as large cytoplasmic granules.

Fig. 7. EGF and TGF\(\alpha\) induction of TGF\(\alpha\) mRNA in BALB/MK cells. Following 48 h in medium lacking EGF, fresh medium without EGF (control) or with 10 ng/ml of either EGF or TGF\(\alpha\) was then added to the cells for 4 h at 37°C. Poly(A)* RNA was purified by oligo-dT affinity chromatography, and Northern analysis was performed using the labeled TGF\(\alpha\) probe as described in "Materials and Methods." RNA isolated from SW620 cells was used as a positive control.

nals provided by high extracellular concentrations of calcium. Although both calcium and TGF\(\beta\) are inhibitory to BALB/MK cells, these studies demonstrate that the two molecules utilize different mechanisms to achieve growth arrest. Calcium induces terminal differentiation of BALB/MK cells (20), while TGF\(\beta\) has no effect on the expression of epithelial differentiation markers. These results differ from those of Masui et al. (18), who found that TGF\(\beta\) treatment resulted in terminal squamous differentiation of human bronchial epithelial cells. Variation in the response of different cell types to TGF\(\beta\) has been demonstrated previously (2). In this study, we provide evidence that TGF\(\beta\) can uncouple inhibition of proliferation and induction of terminal differentiation, thus implicating a possible role for TGF\(\beta\) in the maintenance of the nonproliferative state of epithelial cells. Thus, cells in the basal layer of the skin could be growth arrested without having to enter the terminal differentiation program.

To further address the mechanism of TGF\(\beta\) growth inhibition, the effects on EGF binding were evaluated. TGF\(\beta\) did not alter the interaction of EGF with its cell surface receptor, nor did TGF\(\beta\) affect the internalization of receptor-ligand complexes. These results are in contrast to studies in fibroblastic cells. Assaian et al. (33) have observed a transient decrease followed by an increase in EGF receptors on normal rat kidney fibroblasts at 4–6 h after the treatment of confluent monolayers with TGF\(\beta\) in 10% fetal bovine serum. Massague has reported a rapid and sustained decrease in high affinity EGF receptors in sparse cultures of NRK cells in 0.2% fetal bovine serum (32). Although these investigators are evaluating cells at different growth states under different experimental conditions, it appears that in these fibroblastic cells TGF\(\beta\) exerts some of its effects at the level of the EGF receptor. Massague's group, however, has shown that TGF\(\beta\) does not alter EGF binding in nontransformed (mink lung cells) or transformed (A431) epithelial cells (32, 34). In mink lung cells, this group has demonstrated that the antiproliferative effect of TGF\(\beta\) was not due to an alteration of EGF receptor affinity, number, or postreceptor signaling as measured by phosphorylation of the ribosomal subunit S6 (34). It is not known with the BALB/MK cells if TGF\(\beta\) affects EGF receptor function such as autophosphorylation. While Mullerian inhibiting substance, which shares structural and some sequence homology with TGF\(\beta\), blocks EGF receptor autophosphorylation in A431 cells (44) and rat liver, TGF\(\beta\) does not block EGF receptor autophosphorylation in either system.

We have provided evidence that exogenously added TGF\(\alpha\) and TGF\(\beta\) result in opposing effects on the growth of a non-

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* William E. Russell, personal communication.
transformed murine keratinocyte cell line: TGFα stimulates while TGFβ inhibits growth, and TGFβ can overcome EGF/ TGFα stimulatory signals. Further, both these molecules are produced endogenously by the BALB/MK cells. Our results also demonstrate that TGFα expression can be modulated by EGF or TGFα itself. These findings provide evidence that TGFα may play a role in the positive growth control of keratinocytes. While TGFβ does not appear to perturb proximal EGF ligand receptor interactions, its mechanism(s) of action, as well as the regulation of its production, remains to be elucidated. TGFβ is secreted in a latent form by most cells in culture in a manner similar to that demonstrated for the BALB/MK cells in the present study. Activation of the latent form can be achieved by nonphysiological extremes of pH (15) and by selected proteases. We speculate that at high cell density mechanisms are operative such that sufficient amounts of biologically active TGFβ are made available to the cell to overcome the EGF/TGFα stimulatory loop. This possibility is currently under investigation.

As initially defined, TGFs were polypeptides produced by transformed cells that acted in an autocrine manner to enhance the growth of the malignant cell that produced it (45). Subsequently it was found that the partially purified medium that contained what was initially called sarcoma growth factor and later named TGFα also contained another polypeptide, TGFβ (46). In addition to causing rodent fibroblasts to assume the malignant phenotype (including the ability to grow in soft agar), TGFβ was found to inhibit the monolayer and soft agar growth of a wide variety of cells (notably epithelial cells), normal and neoplastic; thus, the understanding of TGFs had to be expanded (2). Subsequently it was found that the partially purified medium that contained TGFα stimulated bone resorption and inhibited formation in vitro. Proc. Natl. Acad. Sci. USA, 83: 2228–2232, 1986.

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