Autonomous Growth of Human Keratinocytes Requires Epidermal Growth Factor Receptor Occupancy


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Abstract

In the studies reported here, we demonstrate that transforming growth factor α (TGF-α) or epidermal growth factor (EGF) is required for the establishment of small colonies of human keratinocytes at clonal densities, but once small (10–15 cells) colonies have formed, the continued growth of these colonies can proceed in the absence of exogenous TGF-α or EGF. Equivalent receptor-binding concentrations of TGF-α and EGF were equipotent in stimulating colony formation. We also demonstrate that the growth of keratinocytes at high densities proceeds in the absence of exogenous peptide growth factors or hormones. The expression of TGF-α mRNA and protein is regulated by both cell density and the presence of exogenous growth factors. The addition of an antibody which blocks the mitogenic effect of mature TGF-α had no effect on the autocrine/paracrine growth of these cells at either density. However, monoclonal antibodies which antagonize ligand activation of the EGF receptor inhibit the autonomous proliferation of keratinocytes at high density and abrogate the exogenous TGF-α/EGF-independent expansion of colonies at clonal density. The results of these experiments are among the first evidence to demonstrate that normal human epithelial cells in culture exhibit autocrine/paracrine-mediated proliferation. Exogenous growth factors initiate colonies of human keratinocytes that become self-perpetuating in culture. Keratinocytes regulate production of the mitogenic ligand, TGF-α, through a density-dependent mechanism, and cell density stringently controls proliferation.

Introduction

TGF-α is a mitogenic polypeptide growth factor with homology to EGF. Both of these peptides can elicit a growth response by binding to the type 1 EGF receptor (Ref. 1; for a review, see Ref. 2). Both mitogens are synthesized as membrane-bound precursor molecules that contain the mature mitogenic sequence within the NH2-terminal extracellular domain (3–5). Although TGF-α was first identified and characterized in virally transformed cells and tumor cell lines, it has subsequently been shown to be expressed in normal skin, pituitary, decidua cells, and other epithelial tissues (6–10). Studies performed with noncleavable membrane-bound precursor forms (pro-TGF-α) of the peptide have demonstrated that the growth factor can be biologically active while anchored to the plasma membrane (11, 12). Expression of TGF-α in the intact normal epidermis has been demonstrated, and levels of TGF-α mRNA and protein are increased in proliferative skin diseases such as psoriasis (6, 13).

Human epidermal keratinocytes (hereafter referred to as keratinocytes) can be isolated and grown in serum-free medium without the use of mesenchymal cell feeder layers when an optimized basal medium supplemented with growth factors, hormones, and pituitary extract is utilized (Refs. 14–16; for a review, see Ref. 17). Although the presence of pituitary extract appears to be beneficial for long term growth, it has been demonstrated that these cells will proliferate at clonal density (25 cells/cm²) and at high density when the medium is supplemented with only defined growth factors and hormones (EGF or TGF-α, insulin or insulin-like growth factor I, and hydrocortisone) (14, 16, 17). Analysis of numerous studies indicates that clonal growth of keratinocytes requires the presence of EGF or TGF-α at the time of initiating cells in culture. However, Shiple et al. (18) demonstrated that both acidic and basic fibroblast growth factors are mitogenic for these cells in defined medium and that the growth of keratinocytes at high density is supported in medium supplemented with only insulin and hydrocortisone. Coffey et al. (6) demonstrated that TGF-α mRNA and protein are expressed in keratinocytes and that TGF-α is autocrine. The results of these experiments suggested to us that expression of TGF-α mRNA and protein could act at high cell densities as an autocrine/paracrine growth factor for keratinocytes in vitro. In this report, the term autocrine activity refers to production of and stimulation by a growth factor which is restricted to the same cell, whereas paracrine activity denotes stimulation of adjacent cells by a producer cell of identical lineage and differentiated state.

Human epidermis is a stratified epithelium which is organized into proliferative and postmitotic compartments. Keratinocytes apposing or in close proximity to the basement membrane constitute the major proliferative cell population, whereas cells located in the mid-
upper layers of the epidermis have ceased replication and are in the process of stratification and terminal differentiation (for reviews, see Refs. 19 and 20). Regulation of cell renewal and loss within epidermis and other epithelia is largely undefined.

Control of proliferation and differentiation locally within the epidermis might serve as an efficient mechanism to regulate tissue homeostasis and cellular mass balance. Functional EGF receptors are expressed in epidermis, and the greatest density of immunoreactive and ligand-binding receptors resides in the basal-most, proliferative compartment (21–23). These data suggest that normal epidermis may autoregulate keratinocyte proliferation, growth arrest, differentiation, and overall cell turnover by expression of mitogenic growth factor(s) such as TGF-α and cognate receptor(s) such as the EGF receptor on the same cell or neighboring cells. The present studies were undertaken to identify and characterize growth factor-mediated and self-propagated replication of cultured keratinocytes and determine whether blockade of the relevant growth factor receptor and/or neutralization of the potential mitogenic ligands interrupts an autocrine/paracrine regulatory loop which mediates proliferation. We also report the results of experiments to further characterize the modulation of TGF-α mRNA and protein expression by cell density and exogenous growth factors and delineate the relative significance of TGF-α gene expression in keratinocyte proliferation. In the experiments reported here, we demonstrate that exogenous TGF-α or EGF is required only for initiation of clonal growth and that formation of larger colonies as well as proliferation in high density keratinocyte culture is independent of exogenous sources of these mitogens and yet dependent on EGF receptor occupancy.

Results

Mitogenic Potency of Human EGF and TGF-α for Keratinocytes. When keratinocytes are removed from stock culture flasks and plated at clonal densities (25–100 cells/cm²), the cells will form colonies in a completely defined, serum-free medium. Previous studies have shown that EGF is required at the time of cell plating (time 0) for colonies to develop (16). We also have demonstrated previously that human recombinant TGF-α is more potent than natural mouse EGF in stimulating clonal growth (24). We designed experiments to determine whether human recombinant EGF and TGF-α were of equal potency in stimulating colony formation and whether or not EGF or TGF-α was required throughout the duration of colony growth.

The results of experiments summarized here show that human TGF-α and EGF are equipotent in stimulating colony formation of keratinocytes initiated in defined medium. We obtained hEGF and the mature form of hTGF-α produced via recombinant expression in bacteria or yeast. To quantitate equivalent receptor binding concentrations of hTGF-α and hEGF, we performed competition binding studies with these peptides and 125I-EGF on human A431 cells (see “Materials and Methods”). In our assay, the binding activity of these peptides was 0.6 mg of EGF eq/mg of TGF-α, which correlates closely with the value of 0.55 obtained by Winkler et al. (25). When the two growth factors were compared over a range of equivalent receptor binding concentrations, both mitogens stimulated clonal growth of keratinocytes, and no difference was observed in potency, indicating that stimulation of clonal growth by either growth factor is mediated by equipotent occupancy of the EGF receptor (Fig. 1A). No colonies were observed in conditions where cells were plated in medium lacking TGF-α or EGF.

Effects of TGF-α or EGF on Keratinocyte Attachment and Colony Formation. TGF-α or EGF is not required for initial plating of keratinocytes at clonal density but is required to initiate the formation of colonies. Keratinocytes were plated in standard medium MCDB 153 supplemented with insulin (5.0 μg/ml) only, and then TGF-α was added at varying times (Fig. 1B). The addition of TGF-α at times up to 36 h stimulated these cells to form colonies, but at 48 h, the addition of TGF-α had no effect. Thus, keratinocytes have the ability to attach and survive for up to 36 h in the absence of TGF-α or EGF. To determine whether TGF-α/EGF was required throughout the incubation period to obtain large colonies, cells were plated into defined medium (containing TGF-α and insulin) and then washed free of TGF-α at various times (Fig. 1C). To assure that mitogenic amounts of TGF-α did not remain after repeated washings of the clonal dish, the last wash was transferred to a separate clonal assay that was initiated in the absence of TGF-α or EGF. No clonal growth developed over a 10-day incubation period (data not shown). Cells washed free of TGF-α 12 h after plating did not form any macroscopic colonies after 10 days of incubation. However, in cultures washed free of TGF-α 5 days after incubation, after small (10–15 cell) colonies had formed, large colonies developed by the termination of the growth period that were nearly equivalent in size to colonies exposed to TGF-α for the entire incubation period. Visual and phase contrast microscopic examination of colonies propagated in the absence of exogenous TGF-α or EGF revealed more densely clustered, tightly associated human keratinocytes within each colony than those cultures in which TGF-α or EGF was added. Within the colonies, the cells grew as a monolayer, and keratinocytes at the periphery of each colony were less migratory and remained closely attached to the expanding colony. Thus, exogenous TGF-α or EGF is required for the initial phase of colony development, which we term clonal initiation, but is not required for the continued growth of these small, founding colonies, a phase which we designate as clonal expansion.

Effect of Antibodies to Mature TGF-α or EGF Receptor on Clonal Initiation and Expansion. Clonal expansion is blocked by two different monoclonal antibodies directed against the EGF receptor but not by two different anti-TGF-α antibodies. Experiments to characterize the biological activity of TGF-α1 antibody in keratinocyte cultures demonstrated that the monoclonal antibody, which recognizes only the mature, biologically active form of TGF-α, blocked the ability of TGF-α to initiate clonal growth of human keratinocytes, whereas the activity of EGF was unaffected (Fig. 2). When colonies were initiated in TGF-α for 5 days, washed free of TGF-α, and then exposed to the TGF-α1 antibody for the remaining 5 days of growth, the ability of initiated colonies to expand was unaffected (data not shown). The latter was true even though the concentration of antibody that was used was 10-fold higher than that required to block clonal initiation. Similar negative results were observed using the R9 antibody to TGF-α. Therefore, secreted mature
forms of TGF-α are unlikely to be involved solely in clonal expansion.

A monoclonal antibody directed against the EGF receptor was characterized for its ability to block clonal initiation and expansion of keratinocytes. LA-1 completely blocked clonal initiation of keratinocytes induced by TGF-α or EGF (data not shown). We have previously demonstrated that the EGF receptor antibody LA-1 specifically neutralizes EGF receptor-dependent proliferation and is not cytotoxic (26). LA-1 completely blocked clonal initiation of keratinocytes induced by EGF or TGF-α (data not shown). When the LA-1 antibody was added to clonal cultures of keratinocytes in which colonies had been initiated in the presence of TGF-α, the antibody also abrogated the autonomous expansion of these colonies (Fig. 3). A separate EGF receptor antibody, MAb 225, also was capable of neutralizing formation of the ligand-receptor complex (27, 28) and exerted inhibitory effects on clonal initiation and expansion similar to LA-1 antibody (data not shown). A high concentration of TGF-α had the ability to overcome the effect of the LA-1 antibody, and large colonies formed after 10 days of incubation (data not shown). Thus, mature TGF-α does not appear to be responsible solely for clonal expansion or is not accessible to antibody neutralization, but EGF receptor occupancy is required for colonies to expand in the absence of exogenous sources of TGF-α or EGF.

Cell Density and EGF Receptor Mediate Autonomous Proliferation. When keratinocytes are plated at higher densities, initiation of autocrine/paracrine growth requires a minimum permissive cell density and duration of exposure to exogenous growth factors to develop
autonomous cell proliferation. To determine whether growth at higher densities required the continuous presence of TGF-α or EGF, cells were plated at either 10^4 cells/cm^2 or 10^5 cells/cm^2 in complete medium for 7 h (an interval sufficient to allow cell attachment) and then washed thoroughly, and the medium was then replaced with standard medium containing no peptide growth factors. As shown in Fig. 4A, cells plated at 10^5 cells/cm^2 do not proliferate in the absence of added growth factors, but cultures initiated at 10^4 cells/cm^2 proliferate logarithmically after a brief lag phase in the absence of exogenous growth factors (Fig. 4B). Addition of the TGF-α, antibody to high density cells growing in the absence of exogenous TGF-α or EGF had no effect on the ability of these cells to proliferate (data not shown), whereas treatment of cells under similar conditions with the LA-1 antibody at various times during the growth period resulted in prompt cessation of growth (Fig. 5). The ability of the LA-1 antibody to block growth factor-independent growth could be reversed by the addition of excess TGF-α to the growth medium (Fig. 5, arrow). Thus, as with the clonal cultures, keratinocytes have the ability to grow in the absence of exogenously added TGF-α or EGF. Under these conditions, insulin is also not required. This autonomous proliferation is not blocked by an antibody directed against mature TGF-α but is blocked by an antibody which is an antagonist for ligand occupancy of the EGF receptor.

We have previously reported that high density (confluent) cultures of keratinocytes containing growth factors express TGF-α mRNA and protein (6). However, high density cultured keratinocytes switched to standard medium express low or undetectable levels of TGF-α mRNA and protein under these conditions. TGF-α or EGF induces accumulation of TGF-α transcript. In other studies, we have shown that EGF mRNA is not expressed by rapidly growing cultured human keratinocytes (29). In light of our current results demonstrating the continuous growth of keratinocytes in the absence of exogenously added TGF-α or EGF, we examined subconfluent and confluent cultures for the presence of TGF-α mRNA and protein.

Exogenous Growth Factors and Growth State Regulate TGF-α Expression. Subconfluent cultures of keratinocytes growing in the absence of polypeptide growth factors contain significantly greater levels of TGF-α mRNA than cells under the same conditions at confluence, when mitotic activity decreases markedly (Fig. 6, Lanes B and D). In both cases, the presence of exogenous growth factors in the medium caused a relative increase in TGF-α mRNA, although this difference is more pronounced in the confluent cultures. Examination of TGF-α protein levels using a TGF-α ELISA assay which detects mature TGF-α protein revealed a similar pattern of expression of the TGF-α protein (Fig. 7). At confluence, in the absence of exogenous growth factors, the medium and the cell extract contained only a small fraction of the amount of TGF-α that was detected in rapidly growing cultures of cells (Fig. 7). In contrast, both rapidly growing and confluent cultures of keratinocytes in the presence of growth factors contained high levels of TGF-α protein. Thus, the expression of TGF-α mRNA and protein in keratinocyte cultures is dependent on both the density of the cells and the presence or absence of exogenous growth factors in the growth medium.

Cell-associated and secreted forms of TGF-α protein are present in cultures of keratinocytes. To confirm that TGF-α peptide is synthesized by human keratinocytes, the cells were metabolically labeled with [35S]cysteine for 22 h, and immunoprecipitation with TGF-α antibody (R9) was performed. Analysis of the cell extract by gel electrophoresis showed that the 6 kilodalton protein was specifically precipitated by the TGF-α antiserum (Fig. 8). The protein presumably corresponds to the fully processed, 50-amino acid human TGF-α. These results indicate that either the R9 antiserum does not recognize higher molecular weight species of TGF-α or these higher molecular weight species are rapidly processed to the mature form during the labeling period and are not present in sufficient amounts to be precipitated and detected.

Discussion
The original concept and formulation of the autocrine hypothesis, regarding the development and maintenance of the transformed phenotype, incorporated the uniformly held belief that normal cells require exogenous
growth factors for proliferation. This premise led to investigations demonstrating that malignant conversion may involve the novel production and utilization of growth factors which autostimulate the transformed cell (Ref. 30; for reviews, see Refs. 31 and 32). In the case of human epithelial cell transformation, the loss of ability to respond to growth inhibitors or differentiation inducers such as transforming growth factor \( \beta \) also has been proposed as a potential defect that would result in uncontrolled proliferation (33, 34).

The results of our previous and current investigations reveal that at least one type of normal human epithelial cell, the epidermal keratinocyte, can proliferate at high cell density in the absence of exogenous polypeptide growth factors (38, 35, 36). The ability of the cells to proliferate in the absence of exogenous TGF-\( \alpha \) or EGF is not inhibited by the addition of an antibody (TGF-\( \alpha \)1) which can block the biological activity of mature TGF-\( \alpha \). However, TGF-\( \alpha \) mRNA is clearly present in high density proliferating human keratinocyte cultures, and material which interacts with TGF-\( \alpha \) antibodies can be detected in the medium conditioned by these cells and in cell lysates. Metabolic labeling studies further demonstrate that TGF-\( \alpha \) is synthesized and present as a cell-associated growth factor in keratinocytes. Moreover, antibodies that act as agonists of the EGF receptor (MAbs LA-1 and 225) block the growth of these cells at high density and abrogate the expansion of colonies at clonal density. In addition, heparin sulfate inhibits the growth of keratinocytes cultured in the absence of exogenous growth factors (18, 35, 36). We have also demonstrated that other growth factors are present in medium conditioned by keratinocytes. One of these growth factors, KAF, whose activity is inhibited by heparin, has been purified and shown to be similar or identical to human AR (36). Like TGF-\( \alpha \), KAF/AR is synthesized as a membrane-bound precursor molecule. Thus, it appears likely that soluble and/or membrane-anchored precursor forms of TGF-\( \alpha \) or KAF/AR may be involved in the growth of these cells at high density.

At clonal density, non-membrane-bound forms of TGF-\( \alpha \) secreted from cells would be rapidly diluted by the large volume of medium (compared to high density culture), and we presume that concentrations of these species would not be sufficient to stimulate significant cell growth. However, membrane-bound species of TGF-\( \alpha \) or KAF/AR might be able to bind and activate the EGF receptor on the same cell or on adjacent cells, resulting in expansion of existing colonies. The latter alternative seems more likely because single human keratinocytes plated at clonal density do not proliferate in the absence of exogenous TGF-\( \alpha \) or EGF. However, once small colonies of tightly apposed epithelial cells have formed, the requirement for exogenous TGF-\( \alpha \) or EGF to perpetuate colony formation no longer exists. These observations suggest a close link between cell-cell interactions, growth factor production, and proliferation. These biological responses also are dependent on cell density and the ability of human keratinocytes to initiate and expand colonies.

![Graph A](image)

**Fig. 4.** Effect of cell density and growth factors on proliferation of human keratinocytes. A, human keratinocytes inoculated into complete medium at a plating density of \( 1 \times 10^5 \) cells/cm\(^2\). Seven h later, cultures were washed and refed complete medium (O) or standard medium (C) daily. Cell counts were performed on the days indicated. The data reported were means of duplicate samples, and the difference was <10%. B, same as in A, except cells were inoculated at a plating density of \( 1 \times 10^6 \) cells/cm\(^2\).

![Graph B](image)

**Fig. 5.** Effect of anti-human EGF receptor monoclonal antibody on the proliferation of human keratinocytes in the absence of EGF or BPE. Human keratinocyte proliferation in the absence of EGF and BPE was determined as described in "Materials and Methods." EGF receptor monoclonal antibody (LA-1, 4.5 \( \mu \)g/ml) was added at 0 (\( \Delta \)), 1 (\( \triangle \)), 2 (\( \square \)), and 3 (\( \triangledown \)) days after the removal of EGF and BPE from the culture medium. No antibody was added to control cultures (O). Values represent the mean of three separate wells ± SEM (bars). Arrow, the mean number of cells present, after 5 days of culture, in wells receiving TGF-\( \alpha \) (200 ng/ml) and MAB LA-1 (4.5 \( \mu \)g/ml) at time 0.
The results are not surprising considering the anatomy, cellular interactions, and topological organization that characterize ectodermal epithelium, in particular, epidermis (37). Cell interactions in other tissues such as bone marrow may be mediated by similar growth control mechanisms. Ankle'saria et al. (38) have examined the biological responses of hematopoietic progenitor cells expressing EGF receptor by membrane-associated TGF-α on neighboring bone marrow stromal cells, and they proposed the term “juxtacrine” stimulation to describe binding of a membrane-anchored growth factor to its receptor on an adjacent cell. Similar mechanisms may be involved in the growth regulation of epidermal keratinocytes.

Human recombinant TGF-α and EGF are equipotent in initiating clones and generating colonies. The founding cells of a clone must be stimulated by exogenous TGF-α during the first 36 h of incubation, or the potential for clonal formation is irreversibly lost. Once founding cells form sufficient progeny to sustain their mitogenic requirements, human keratinocytes composing these colonies will continue to proliferate in the absence of exogenous TGF-α or EGF. Previous investigation has shown that TGF-α is more efficient than EGF in stimulating growth of large-sized colonies or “megacolonies” (i.e., 24 days of culture, 15–20 cm² surface area) and enhancing cell migration within the colony (39). However, these studies were conducted in serum-containing medium with a lethally irradiated mesenchymal cell feeder layer, and autocrine/paracrine growth behavior could not be characterized.

When human keratinocytes proliferate at high density in the absence of exogenous polypeptide growth factors (standard medium), the level of TGF-α mRNA is regulated by the state of confluence of the cells. In subconfluent cultures of cells, the levels of TGF-α mRNA are significantly greater than in the same cultures at confluence, when proliferation decreases dramatically. The levels of TGF-α mRNA in confluent cultures can be enhanced significantly by the addition of exogenous TGF-α or EGF, as previously reported (6). The mechanism by which TGF-α mRNA levels are down-regulated at confluence in human keratinocyte cultures proliferating in standard medium is not clear. Nor is it established whether the decrease in TGF-α levels is one of the primary events that inhibits cellular proliferation as confluence is reached. However, studies have shown that TGF-α-transfected mesenchymal cells which express and secrete large amounts of TGF-α protein attain 2–4-fold higher saturation densities than control cell lines (40). Interestingly, TGF-α antibody only partially inhibits this response.

Although it has been postulated that TGF-α could act as an autocrine/paracrine growth factor for human keratinocytes, it has not been possible to block autonomous proliferation of these cells with specific TGF-α antibodies. However, the expression of TGF-α peptide and mRNA coupled with the ability of specific EGF receptor monoclonal antibodies to block the autonomous growth of these cells suggests that TGF-α, perhaps present as multiple secreted and membrane-anchored species, acts as an autocrine/paracrine growth factor to stimulate mitogenesis of human keratinocytes. These results suggest that epithelial cells, such as human keratinocytes, with a constitutively active but stringently regulated state of proliferation in vivo, exert growth control locally to mediate replication, quiescence, and differentiation. The epidermis also exhibits the capacity to increase its mitotic activity dramatically during regenerative states following wounding and in hyperproliferative diseases such as psoriasis (for reviews, see Refs. 41 and 42). In this regard, we have shown that involved but not uninvolved psoriatic epidermis expresses significantly greater levels of TGF-α mRNA and protein and AR mRNA (13, 43). Overexpression of TGF-α targeted to epidermis of transgenic mice also induces hyperproliferation of keratinocytes and thickening of epidermis (44).

Expression and regulation of a growth factor such as TGF-α and its cognate receptor, the EGF receptor, may
enable the epidermis to autoregulate proliferation and cellular mass. TGF-α and EGF receptor also may mediate epidermal regeneration and trigger hyperproliferative responses. The regulated expression and activation of autocrine/paracrine growth factors and their relevant receptors may be biological functions intrinsic to normal epithelial cells. In addition, it should not be viewed as unusual that many epithelial-derived tumor cell lines in culture constitutively produce autocrine growth factors. Transformed cells may alter these normal growth-regulatory mechanisms to acquire a proliferative advantage. By comparison, mesenchymal cells such as fibroblasts are not as mitotically active in normal resting tissues and do not exhibit the distinctive features of cell-cell interactions and cooperativity that are hallmarks of ectodermal epithelium (37). Mesenchymal cells have more stringent requirements for exogenous growth factors to replicate and to initiate autocrine proliferative pathways (18, 45–47).

These studies demonstrate the ability of normal ectodermal epithelial cells to express growth factors that mediate autonomic proliferation in vitro. TGF-α and other mitogenic peptide growth factors (e.g., AR) may be produced locally within a tissue, such as epidermis, to sustain cell renewal and modulate replication. Abnormal expression or activation of growth factors and/or their receptors may contribute to epidermal hyperplasia, atrophy, and aging as well as malignant transformation.

Materials and Methods

Reagents. Human recombinant TGF-α was the mature 50-amino acid peptide isolated from Escherichia coli W3110 containing the plasmid pTE5 as described (4). Human recombinant EGF derived from yeast was kindly provided by Drs. P. Valenzuela and C. George-Nascimento (Chiron, Emeryville, CA) or obtained from Amgen (Thousand Oaks, CA). Mouse EGF, receptor grade, was obtained from Collaborative Research (Bedford, MA). Human recombinant basic fibroblast growth factor was a gift from Dr. J. Abraham (Scios-Nova, Mountain View, CA). Antibodies recognizing TGF-α were prepared and isolated as described (MAb TGF-α1) (48) or obtained from Dr. R. Hanks (antibody R9; Triton Bioscience, Alameda, CA). Monoclonal antibodies recognizing the EGF receptor (MAb LA-1 and 225) were obtained commercially (Upstate Biotechnology, Lake Placid, NY) or as a gift from Drs. H. Masui and J. Mendelsohn, respectively. Bovine insulin, hydrocortisone, ethanolamine, and phosphoethanolamine were obtained in the highest grade from Sigma (St. Louis, MO).

Culture of Normal Human Keratinocytes. The high amino acid formulation of medium MCDB 153 was prepared by adding the appropriate amount of a 100X concentrated amino acid stock to the basal medium as previously described (15) or was obtained from Clonetics Corp. (San Diego, CA) as keratinocyte basal medium. Primary cultures of human keratinocytes were isolated from foreskin by the trypsin float technique as previously described (16). Stock cultures were maintained in an actively growing state in high amino acid medium MCDB 153 supplemented with 0.2% (v/v) BPE, EGF (10 ng/ml), insulin (5 μg/ml), hydrocortisone (5 × 10⁻² M), ethanolamine (1 × 10⁻⁴ M), and phosphoethanolamine (1 × 10⁻⁴ M), or in KBM supplemented with EGF, insulin, BPE, and hydrocortisone at the same concentrations. This growth medium is hereafter referred to as “complete” medium. Complete medium without BPE, EGF, and insulin is referred to in the text as “standard” medium. Secondary and tertiary cultures of keratinocytes were used for growth assays and preparing conditioned medium for determination of TGF-α content.

Clonal growth assays were performed as previously described (16). Briefly, 500 cells were inoculated into 60-mm dishes containing MCDB 153 medium and the designated additives. Growth factors or antibodies were added to selected cultures. If growth factors were removed from the culture medium, cultures were washed repeatedly before switching to medium without growth factors. Cultures were fixed at the times indicated, stained with crystal violet, and photographed. Cell density of high density cultures was determined by trypsinization and cell enumeration as previously described (16).

RNA Isolation and Northern Blot Analysis. Polyadenylated mRNA from keratinocyte cultures was isolated by the method of Schwab et al. (49). mRNA was separated by electrophoresis in 1.2% agarose-formaldehyde gels, and Northern blotting was performed as described (50). The TGF-α clone used as a probe is a human 1.27-kilobase complementary DNA encompassing the entire open reading frame as well as 0.79 kilobase of flanking
3’ untranslated sequence (4). The probe was subcloned into plasmid sp65 and labeled with [α-32P]UTP. For all Northern blots, hybridization with a complementary DNA probe (1815) for the constitutively expressed cyclophilin gene (51) was used to assure equivalent loading and transfer of RNA (52).

**TGF-α Protein.** Conditioned medium from keratinocyte cultures was collected, clarified by centrifugation, dialyzed against 0.1 M acetic acid, and lyophilized. Cell extracts also were processed, and amounts of TGF-α were measured by a TGF-α-specific double sandwich ELISA as previously described (6, 48).

**Metabolic 35S Labeling.** Keratinocytes were grown to near-confluence in 60-mm dishes containing complete MCDB 153 medium. The cells were washed three times with phosphate-buffered saline and replaced with 1.5 ml of cysteine-free complete MCDB 153 medium to which 160 µCi/ml L-[35S]cysteine (NEN Research, Boston, MA) were added. The cultures were incubated for 22 h at 37°C. Cells were removed with a rubber policeman and collected in phosphate-buffered saline containing 0.5 mM phenylmethylsulfonyl fluoride.

**Immunoprecipitation and Gel Electrophoresis.** The procedure was similar to that previously described except that immunoprecipitation was performed with the R9 TGF-α antibody (48). The samples were diluted and incubated with R9 antibody or preimmune serum overnight at 4°C. Antigen-antibody complexes were isolated by Protein A-agarose beads. After removal of beads, the samples were analyzed by electrophoresis in denaturing 15% polyacrylamide gels. Prestained molecular weight markers were purchased from BRL. The gels were fixed, treated with Enhance (NEN), dried, and exposed to Kodak XAR-2 film at ~70°C.

**125I-EGF-binding Competition Assay.** The 125I-labeled EGF radioreceptor assay was performed using A431 cells as described previously (53). The relative competing activity of human recombinant EGF and TGF-α was determined by performing binding assays over a wide range of concentrations.

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**References**


