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TGF-β and retinoic acid: regulators of growth and modifiers of differentiation in human epidermal cells

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In the epidermis of skin, a fine balance exists between proliferating progenitor cells and terminally differentiating cells. We examined the effects of TGF-βs and retinoic acid (RA) on controlling this balance in normal and malignant human epidermal keratinocytes cultured under conditions where most morphological and biochemical features of epidermis in vivo are retained. Our results revealed marked and pleiotropic effects of both TGF-β and RA on keratinocytes. In contrast to retinoids, TGF-βs acted on mitotically active basal cells to retard cell proliferation. Although withdrawal from the cell cycle is a necessary prerequisite for commitment to terminal differentiation, TGF-βs inhibited normal keratinization in suprabasal cells and promoted the type of differentiation commonly associated with wound-healing and epidermal hyperproliferation. The actions of TGF-βs and RA on normal keratinization were synergistic, whereas those on abnormal differentiation associated with hyperproliferation were antagonistic. These observations underscore the notion that environmental changes can act separately on proliferating and differentiating cells within the population. Under the conditions used here, the action of TGF-βs on human keratinocytes was dominant over RA, and TGF-βs did not seem to be induced as a consequence of RA treatment. This finding is consistent with the fact that RA accelerated, rather than inhibited, proliferation in raft cultures. Collectively, our data suggest that the effects of both factors on epidermal growth and differentiation are multifaceted and the extent to which their action is coupled in keratinocytes may vary under different conditions and/or in different species.

Introduction

The epidermis manifests its protective function by building an extensive cytoskeleton, the unique feature of which are 10-nm keratin filaments. In basal cells, these filaments are composed of two keratins K5 (58 kd) and K14 (50 kd) (Nelson and Sun, 1983). The four to eight layers of suprabasal spinous cells are postmitotic, but metabolically active. These cells manufacture two new keratins, K1 (67 kd) and K10 (56.5 kd) (Fuchs and Green, 1980). Spinous cells also make cornified envelope proteins, such as involucrin (Rice and Green, 1979). As spinous cells enter the granular layer, they stop making keratin and envelope proteins and produce filaggrin, a protein which may be involved in keratin filament bundling into macrofibrils (Dale *et al.*, 1978). As the cell becomes permeable, a calcium influx activates epidermal transglutaminase, which biochemically cross-links the envelope proteins into a cage, encapsulating the macrofibrils (Rice and Green, 1979). As lytic enzymes are released, all vestiges of metabolic activity terminate, and the resulting flattened squames are merely cellular skeletons, chockfull of macrofibrils of keratin filaments.

A prerequisite for commitment of an epidermal cell to terminally differentiate is cessation of cell growth (Pittelkow *et al.*, 1986; Albers *et al.*, 1987; Dover and Potten, 1988; Barrandon *et al.*, 1989). Major regulators in this process are TGF-βs, which act at concentrations as low as 1 ng/ml to inhibit epidermal proliferation (Shipley *et al.*, 1986; Kopan *et al.*, 1987; Coffey *et al.*, 1988). Under normal conditions in vivo (Thompson *et al.*, 1989) and in vitro (Shipley *et al.*, 1986; Glick *et al.*, 1989; Bascom *et al.*, 1989), TGF-β1 messenger RNAs (mRNAs) are made in terminally differentiating epidermal cells. TGF-βs can be secreted in latent form (Lyons *et al.*, 1988; Miyazono *et al.*, 1988; Glick *et al.*, 1989), and hence TGF-β mRNA expression may not necessarily correlate with active TGF-β production. This caveat aside, TGF-βs may maintain cessation of DNA synthesis in suprabasal layers. Developmental studies support this notion and show that TGF-β2 and a TGF-β-related gene, Vgr-1, are expressed coincident with stratification and keratinization in developing mouse epidermis (Lyons *et al.*, 1989).

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It has been difficult to assess whether TGF-βs control the biochemistry of epidermal keratinization. One reason is that the action of TGF-βs is largely reversible within a 48-h period, even though terminal differentiation seems to be irreversible (Shipley *et al.,* 1986). In addition, TGF-βs do not induce K1/K10 expression, associated with keratinization (Kopan *et al.,* 1987; Coffey *et al.,* 1988). In one study, TGF-βs actually inhibited K1 synthesis (Mansbridge and Hanawalt, 1988). Interestingly, Mansbridge and Hanawalt (1988) also showed that TGF-βs enhanced expression of K6 (56 kd) and K16 (48 kd), expressed transiently in suprabasal cells during wound healing (Mansbridge and Knapp, 1987) and constitutively in cultured cells (Kopan and Fuchs, 1989) and in hyperproliferative diseases (Weiss *et al.,* 1984; Stoler *et al.,* 1988). However, since this and other studies on TGF-βs have been conducted with epidermal cells cultured on plastic, under conditions where many of the differentiative features of epidermis are not retained, the relation between TGF-βs and epidermal differentiation has remained unclear.

Although the possible role of TGF-βs in keratinization has only recently gained attention, it is well known that other extracellular regulators, namely retinoids, have a profound negative effect on epidermal differentiation both in vivo (Wolbach, 1954) and in vitro (Yuspa and Harris, 1974; Fuchs and Green, 1981). When vitamin A is removed from serum, cultured epidermal cells display many characteristics of terminal differentiation (Fuchs and Green, 1981). These features include stratification, cell adhesiveness, reduced cell motility, expression of K1 and K10, and filaggrin expression (Fuchs and Green, 1980; Watt and Green, 1982; Kim *et al.,* 1984; Fleckman *et al.,* 1985). In contrast, when epidermal cells are cultured at an air–liquid interface so that they are not in direct contact with vitamin A-containing medium, supplemental addition of retinoids to the medium can markedly inhibit many of the features of terminal differentiation, including K1/K10 and K6/K16 expression (Kopan *et al.,* 1987; Kopan and Fuchs, 1989). Many of these effects extend to the level of mRNA expression (Fuchs and Green, 1981; Kim *et al.,* 1984; Kopan and Fuchs, 1989), and at least some may be at the transcriptional level (Stellmach and Fuchs, 1989).

In the past year, an interesting link between RA and TGF-βs was uncovered (Glick *et al.,* 1989). Induction of TGF-β2 mRNA was found in mouse keratinocytes treated with elevated concentrations ($\sim 10^{-6}$ M) of RA in vitro or with topical application of retinoids to skin (Glick *et al.,* 1989). In mouse keratinocytes, these retinoid concentrations are sufficient to inhibit proliferation, and TGF-β2 seemed to mediate these antiproliferative effects (Glick *et al.,* 1989). Since RA elicits some changes in gene expression that TGF-βs do not, its action may sometimes extend beyond mere induction of TGF-β2 expression (Glick *et al.,* 1989). However, because TGF-βs and RA elicit certain effects on epidermal differentiation that are antagonistic (Kopan and Fuchs, 1989; Mansbridge and Hanawalt, 1988), TGF-βs may elicit effects that extend beyond those of RA.

To further explore the relationship between TGF-βs, retinoids, and epidermal differentiation, we utilized an in vitro culture system where human epidermal cells are cultured at the air–liquid interface on a collagen lattice containing fibroblasts (Flaxman and Harper, 1975; Lillie *et al.,* 1980; Asselineau *et al.,* 1986; Kopan *et al.,* 1987). This system enables most of the differentiated properties of epidermis to take place in vitro, and consequently, it is possible to examine TGF-β– and RA-mediated action on epidermal cells at various stages of growth and differentiation. Using [^{3}H]-thymidine labeling, immunoblot analysis, immunohistochemistry, and [^{35}S]-methionine labeling, we examined the effects of TGF-βs and/or RAs on tissue morphology, cell proliferation, and terminal differentiation. Our results have revealed pleiotropic, synergistic, and antagonistic effects of these two regulators of keratinocyte growth and differentiation.

Results

TGF-β alters the morphology of human epidermal cultures

Human epidermal cells cultured on a floating lattice of collagen and 3T3 fibroblasts stratified extensively and showed a gradual series of morphological changes characteristic of terminal differentiation (Figure 1A; Asselineau *et al.,* 1986; Kopan *et al.,* 1987). Most phases of normal morphology were preserved at 4 pM TGF-β2, although few granular cells were seen (Figure 1B). At 40 pM TGF-β2, marked morphological changes were observed at most stages of epidermal differentiation (Figure 1C). Spinous cells displayed keratin pearls (see arrow in C), vacuoles (arrowheads in C), and pyknotic nuclei, all of which are often seen in squamous cell carcinomas of skin (see e.g., Stoler *et al.,* 1988). Rafts grown in the presence of 40 pM TGF-β2 showed no granular layer and only a very thin stratum corneum (s.c.) layer. Despite these

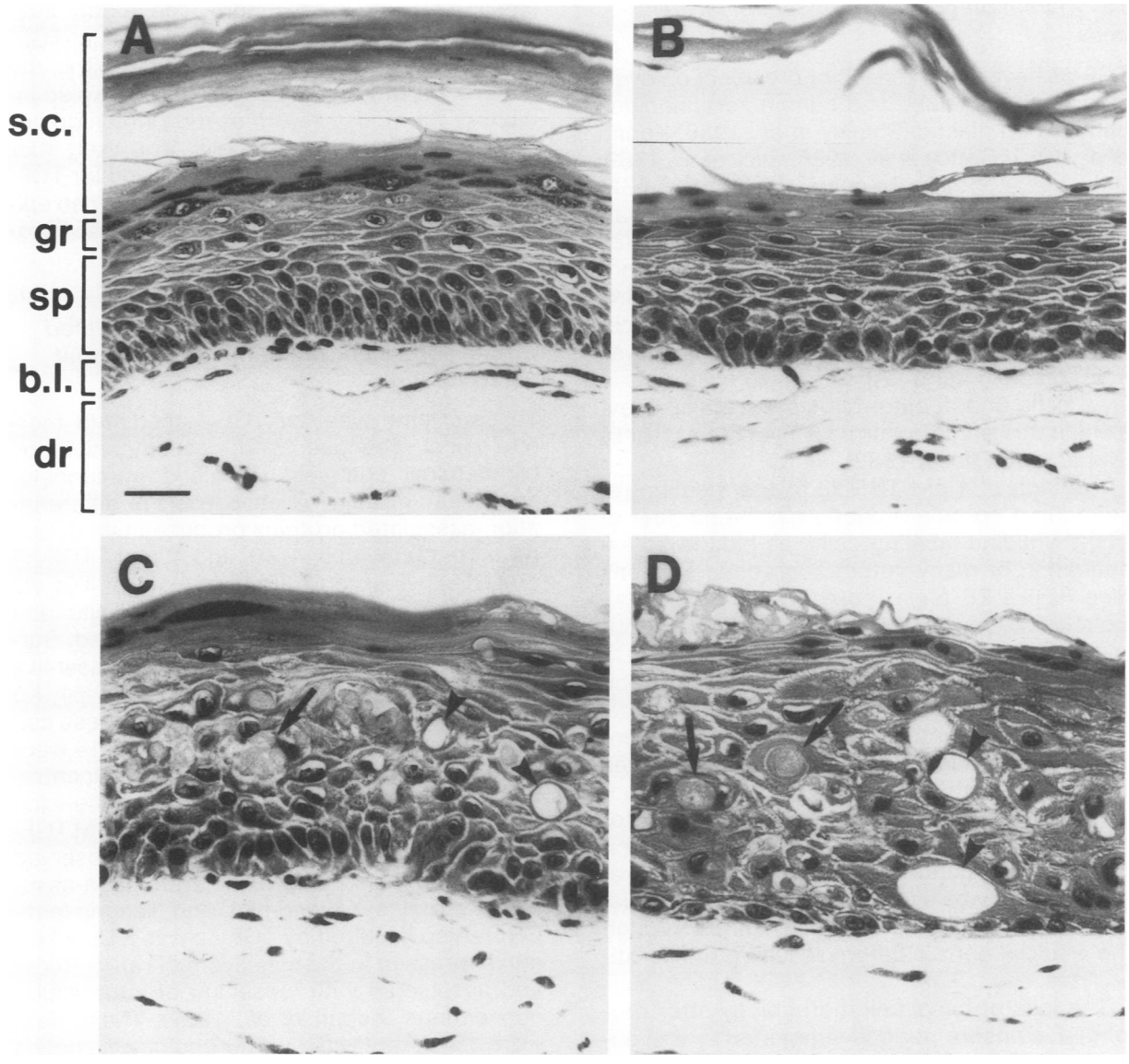


Figure 1. **Effects of TGF-β2 on morphology of human epidermal differentiation.** Human epidermal keratinocytes were cultured at the air–liquid interface for 3 wk in the presence of 0 pM TGF-β2 (A), 4 pM TGF-β2 (B), 40 pM TGF-β2 (C), and 400 pM TGF-β2 (D). Rafts were fixed in Carnoy's solution, embedded in paraffin, and sectioned (5 μm). Sections were stained with hematoxylin and eosin and visualized by light microscopy. Arrows indicate keratin pearls and arrowheads indicate vacuoles. s.c., stratum corneum layers; gr, granular layers; sp, spinous layers; b.l., basal layer; dr, artificial dermis (lattice of collagen and fibroblasts). Bar = 27.5 μm.

morphological aberrations in the differentiating layers, basal-like cells were still seen in many regions of the raft cultures. At 400 pM TGF-β2, most vestiges of a basal-like cell population disappeared, and keratin pearls, vacuoles, and pyknotic nuclei were even seen in the innermost epidermal layer (Figure 1D). The dramatic alterations in morphology appeared to be most prominent when cultures were grown for prolonged periods at elevated concentrations of TGF-β2: cultures grown for 3 wk in normal medium, followed by growth in the presence of 400 pM TGF-β2 for 3 d were much less affected (not shown).

To determine whether effects of TGF-β2 on epidermal differentiation extend to other TGF-βs, we repeated these studies using TGF-β1 instead of TGF-β2. Our results were indistinguishable from those obtained with TGF-β2 (data not shown).

TGF-βs inhibit growth of basal epidermal cells

TGF-βs have a marked inhibitory effect on overall growth of mouse and human keratinocytes cultured on plastic (Shipley *et al.,* 1986; Kopan *et al.,* 1987; Coffey *et al.,* 1988; Glick *et al.,* 1989; Rollins *et al.,* 1989). To examine effects of TGF-βs on proliferation of human keratinocyte raft cultures, we labeled untreated and TGF-β2–treated raft cultures with [^{3}H]-thymidine before fixation and autoradiography (Figure 2). Labeling of nuclei was largely confined to the innermost layer, and with no TGF-β added, labeling was appreciable (Figure 2A). Similar to epidermis in vivo, cells undergoing mitosis were also prevalent in this single, innermost layer of basal cells (Kopan and Fuchs, 1989).

Addition of 4 pM TGF-β2 to the medium resulted in a notable reduction in the number of [^{3}H]-thymidine labeling cells, as judged by examination of many autoradiographed sections (see Figure 2B for a representative example). Concentrations of 40 pM TGF-β2 inhibited DNA synthesis to an even greater extent (Figure 2C), and at 400 pM TGF-β2, very few labeled epidermal cells were seen (Figure 2D). The retardation in DNA synthesis was much more prominent after prolonged treatment of raft cultures with TGF-βs than after 3 d treatment (not shown). That much higher levels of TGF-βs for much longer times are necessary to inhibit growth of raft cultures versus submerged keratinocytes may be a reflection of our culture system, which requires diffusion of TGF-βs through the artificial dermis before reaching the keratinocytes.

The loss of basal cell morphology after prolonged exposure periods suggested that eventually the effects of TGF-βs may become irreversible. To examine this possibility, we took 3 wk TGF-β2–treated (40–400 pM) raft cultures and then either labeled directly with [^{3}H]-thymidine or withdrew TGF-β2 for 1 wk before labeling. Surprisingly, even after rafts were exposed to 400 pM TGF-β2 for 3 wk, an increase in DNA synthesis was observed after return to normal medium (not shown). However, the reversibility was limited and was less than under milder conditions. These data are consistent with the morphological appearance of differentiating cells in the basal layer after prolonged treatments with high levels of TGF-βs.

While high concentrations of TGF-βs showed marked inhibition of epidermal growth, fibroblast growth in the collagen lattices was not inhibited, as judged by no loss in [^{3}H]-thymidine labeling after 2–3 wk culture. These data indicated that the growth inhibitory effects of TGF-β2 were specific for the keratinocytes within the culture. Furthermore, since TGF-βs are also inhibitory for mouse keratinocytes, which do not require fibroblast feeder cells, it is likely that the growth inhibitory effects observed for TGF-βs were administered directly to the human epidermal cells, rather than indirectly via the fibroblasts.

TGF-βs enhance differentiation associated with hyperproliferation and inhibit normal differentiation

To determine whether TGF-β–mediated morphological changes were accompanied by biochemical changes, we used immunoblot analysis to examine relative levels of differentiation-associated proteins produced by raft cultures treated with 0, 4, 40, and 400 pM TGF-β2 or TGF-β1 (Figure 3). Immunoblots of TGF-β2 and TGF-β1 experiments were very similar, and hence only data for TGF-β2 are provided. Surprisingly, despite the absence of basal-like morphology at 400 pM TGF-β2 or TGF-β1, K5 and K14 were still readily detected in these cultures, and in fact, the levels of K5 and K14 were somewhat higher at elevated TGF-β concentrations [compare anti-hK14/anti-hK5 in lane 1 (no TGF-β2) with lanes 2–4 (4, 40, and 400 pM TGF-β2)]. In contrast, a marked decline was observed in the expression of two differentiation-associated proteins, K1 (anti-hK1) and filaggrin (anti-hFIL). Also inhibited by TGF-βs was K13, a keratin expressed in the s.c. layers of raft cultures and associated with loosening of intercellular connections (Kopan *et al.,* 1987). These data were consistent with our morphological studies showing that the latter stages of differentiation were most sensitive to TGF-βs.

While some biochemical features of terminal differentiation were affected by TGF-βs, others were not. In particular, we discovered that expression of involucrin, a cornified envelope protein, showed no sensitivity to TGF-β (anti-hINV, lanes 1–4). Moreover, the concentrations necessary to influence some of the biochemical indicators of differentiation were higher than those necessary to reduce keratinocyte growth. Collectively, our data indicated that expression of some, but not all, biochemical markers of terminal differentiation were inhibited by elevated levels of TGF-βs and in a dose-dependent fashion. These results confirmed and extended previous studies involving human keratinocytes cultured on plastic (Mansbridge and Hanawalt,

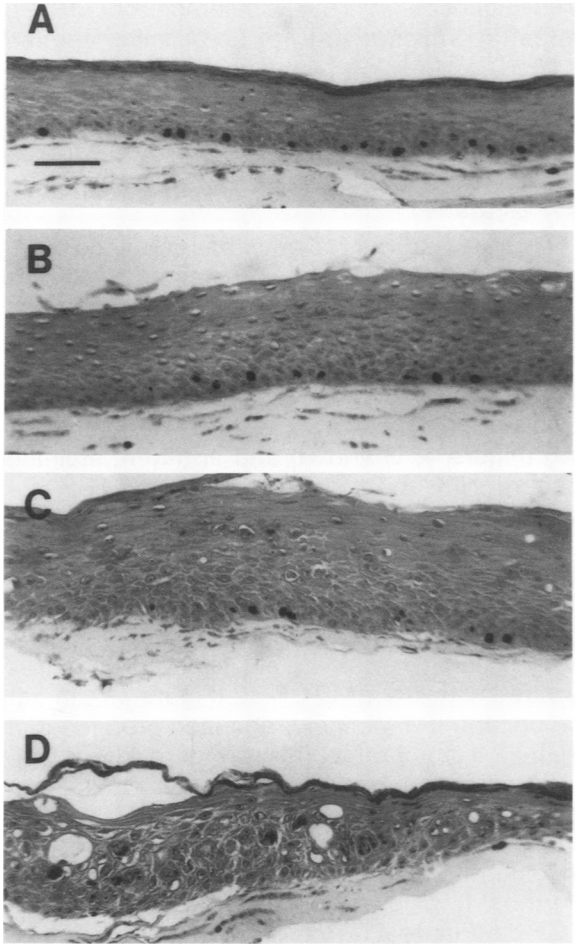


Figure 2. **Effects of TGF-β2 on proliferation of human epidermal raft cultures.** Keratinocytes were cultured for 3 wk at the air–liquid interface in the presence of 0 pM TGF-β2 (A), 4 pM TGF-β2 (B), 40 pM TGF-β2 (C), and 400 pM TGF-β2 (D). Rafts were labeled with [^{3}H]-thymidine for 2 h, washed extensively, fixed in Carnoy's solution, and embedded in paraffin. Sections (5 μm) were deparaffinized, dehydrated, and exposed to Kodak NTB2 emulsion for 3 wk. Sections were then developed and stained with hematoxylin and eosin. Black grains indicate nuclear labeling. Bar = 68.8 μm.

1988). They underscored the fact that not only were exogenously added TGF-βs ineffective at inducing differentiation in vitro, they actually inhibited it, providing that the concentration was sufficiently high.

Among the most interesting of TGF-β–mediated changes was the appreciable increase in expression of K16 and K6 (Figure 3, anti-hK16 and anti-hK6, respectively). This finding confirms and extends previous protein studies by Mansbridge and Hanawalt (1988). Moreover, coupled with our [^{3}H]-thymidine studies, our data clearly indicate that K6 and K16 expression can be uncoupled from hyperproliferation and that changes in their expression more likely arise from environmental rather than proliferative changes per se. This notion is consistent with recent studies by Kopan and Fuchs (1989) demonstrating an enhancement in proliferation and suppression of K6/K16 expression in retinoid-treated raft cultures. It is also consistent with recent studies by Schermer *et al.* (1989) showing that DNA synthesis inhibitors did not block synthesis of K6/K16 in rabbit corneal epithelial cells.

In addition to examining effects of TGF-βs on expression of epidermal markers, we investigated fibronectin, a protein whose expression has been shown to be elevated upon treatment of fibroblasts with TGF-β (Ignotz and Massague, 1987; Massague, 1987). Indeed, fibronectin levels were greatly elevated in raft cultures treated with TGF-βs (anti-hFN, lanes 1–4). Since both fibroblasts and epidermal cells synthesize fibronectin, immunoblot analysis alone was not sufficient to determine which cell type in the raft cultures was responsible for elevated fibronectin expression.

Exploring the relations between the morphological and biochemical changes induced by TGF-β2

To examine the possible correlation between TGF-β–induced morphological changes and changes in keratin, involucrin, and filaggrin, we stained sections of TGF-β-treated floating cultures with antisera specific for each of these epidermal markers. We then compared the patterns of indirect immunohistochemical staining of TGF-β-treated rafts with those generated by untreated rafts and by epidermis from human skin (Figure 4).

Anti-human K5 antisera (anti-hK5; Figure 4A) and anti-human K14 antisera (not shown) stained the basal layer of human skin, with only weak staining of the suprabasal layers. Anti-human K1 antisera (Figure 4E) and anti-K10 (not shown) stained only keratinizing layers of skin. The pattern of anti-hK5 and anti-hK1 staining in raft cultures was somewhat different from that of human skin. Hence, anti-hK5 stained all but the s.c. layers (Figure 4B), whereas anti-hK1 often showed strong staining only in upper keratinizing layers (Figure 4F). The differences in patterns seem to arise from three phenomena: 1) the levels of K14/K5 relative to K1/K10 are much higher in raft cultures than in epidermis (Kopan *et al.*, 1987); 2) K1/K10 expression seems to cause an increase in filament aggregation, possibly leading to antigen masking of K14/K5 in

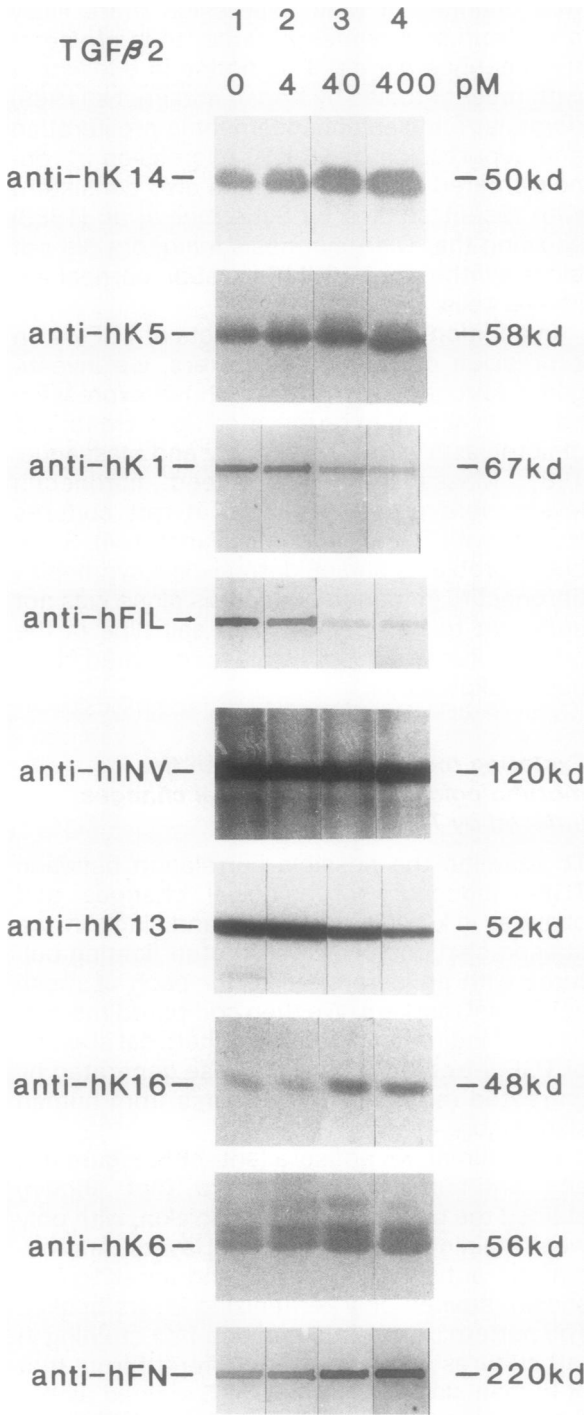


Figure 3. **Immunoblot analysis of proteins from rafts cultured in medium containing 0–400 pM TGF-β2.** Keratinocytes were cultured at the air–liquid interface for 3 wk in the presence of 0 pM TGF-β2 (lane 1), 4 pM TGF-β2 (lane 2), 40 pM TGF-β2 (lane 3), and 400 pM TGF-β2 (lane 4). Triton-insoluble and triton-soluble protein extraction was conducted as described in Materials and methods. For each set of TGF-β-treated cells, equal protein samples were taken and resolved by electrophoresis through 8.5% polyacrylamide gels. Sample sizes and triton-insoluble vs. triton-soluble protein samples were chosen according to the antibody used for immunoblot analysis. Immunoblot analysis was performed as described in Materials and methods. For K1, filaggrin and fibronectin, posttranslational processing occurs in vivo, and hence, these samples were slot-blotted to nitrocellulose paper rather than blotted from gels. Antibodies used are given at left. Estimated molecular masses of immunoreactive bands are indicated at right. Since filaggrin is synthesized as a precursor and then processed in a multistep fashion, no molecular mass is indicated. Samples used for analyses were as follows: 0.2 μg triton-insoluble proteins for anti-hK14; 1 μg triton-insoluble proteins for anti-hK5 and anti-hK6; 5 μg of triton-insoluble proteins for anti-hK1, anti-hK13, anti-hK16, and anti-involucrin (anti-hINV); 15 μg of triton-insoluble proteins for anti-filaggrin (anti-FIL); 5 μg triton-soluble proteins for anti-fibronectin (anti-hFN). Within a given set of samples, color development to measure antibody binding was stopped at the same time.

vivo (Eichner *et al.*, 1986; Coulombe *et al.*, 1989), a feature which might not be prominent when K1/K10 expression is low and K5/K14 expression is high; and 3) proteolytic processing of K5/K14 occur in the latter stages of terminal differentiation (Fuchs and Green, 1980), most likely leading to absence of anti-hK5 staining in s.c. layers.

For raft cultures grown in the presence of 40 or 400 pM TGF-β2, anti-hK5 stained all epidermal structures, except the keratinized pearls (Figure 4, C and D, respectively). Staining in the outermost layers was consistent with the absence of granular and s.c. layers in TGF-β2–treated cultures. The absence of anti-hK1 staining (Figure 4, G and H) and anti-hFIL staining (Figure 4, K and L) in 40 or 400 pM TGF-β2–treated cultures was also consistent with the marked inhibition of normal differentiation. In contrast, anti-involucrin staining was still prominent in the keratinizing layers of cultures treated with 40 pM TGF-β2 (Figure 4O), and surprisingly, it was in all layers of cultures treated with 400 pM TGF-β2 (Figure 4P). In addition, anti-hK6 staining remained prominent in TGF-β2 treated raft cultures (Figure 4, S and T), consistent with our immunoblot analyses demonstrating elevated levels of K6/K16 under these circumstances. Moreover, similar to anti-involucrin, anti-hK6 staining extended to the innermost layer of cultures treated with 400 pM TGF-β2 (Figure 4T). Expression of these differentiation-specific markers in the innermost layer of rafts treated with 400 pM TGF-β2 correlated with absence of basal-like morphology and DNA synthesis in these cultures. It also correlated with the reduction in proliferation reversibility seen after prolonged treatment of rafts with TGF-β2.

To determine whether TGF-β2 mediated elevation of fibronectin expression in rafts stemmed from the keratinocytes or the dermal fibroblasts (or both), we stained sections with an anti-fibronectin antisera (Figure 4, U–X). Our results show clearly that staining was most abundant in the fibroblast/collagen lattice of TGF-β2–treated cultures (Figure 4, W and X). Although we cannot rule out TGF-β2–mediated enhancement of fibronectin expression in raft keratinocytes, we can say that if it occurs, it is markedly less than that seen in the dermal fibroblasts.

TGF-β2 and RA can affect keratin expression within 2 d exposure

Previously, a negative effect was observed on both K1/K10 and K6/K16 synthesis in 3-wk raft cultures grown in the presence of 1×10^{-7} to 1×10^{-6} M RA (Kopan *et al.,* 1987; Kopan and Fuchs, 1989; Asselineau *et al.,* 1989). To determine whether the TGF-β– and RA-mediated changes in keratin expression occur over the entire 3-wk period, or alternatively, whether they can be induced within a much shorter time, we cultured rafts for 18 d and then exposed them for 48 h to either 400 pM TGF-β2, 1×10^{-6} M RA, or a combination of TGF-β2 and RA. We then radiolabeled cultures with [^{35}S]-methionine, and isolated and resolved keratins by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and autoradiography (Figure 5A). Interestingly, biosynthesis of K1 (67 kd) was completely inhibited after 2 d exposure to TGF-β2 (lane 3), just as it was when TGF-β2 was added to cultures from the beginning (lane 2). An increase in synthesis of K16 (48 kd) and a decrease in K13 (52 kd) were also observed after short exposure of differentiating cultures to TGF-β2 (cf. lane 1, untreated, with lane 3, 48-h post-TGF-β2 addition), although these changes were not as prominent as those seen upon establishment of differentiating cultures in the presence of TGF-β2 (lane 2). Thus, suppression of K1 synthesis appeared to precede enhancement of K16 and suppression of K13 synthesis.

To determine whether retinoids could also exert their effects on keratin synthesis within a relatively short time period, we cultured epidermal rafts for 18 d, followed by exposure to 1×10^{-6} M RA. Suppression of K1 biosynthesis occurred within 2 d of RA treatment (Figure 5B, lane 3). RA-mediated elevation in K13 synthesis also occurred within 2 d after RA exposure. Hence, similar to our observations with TGF-β2, the major changes in keratin biosynthesis elicited by RA took place in cells within a relatively short time frame. Interestingly, morphological changes in raft cultures lagged behind both RA and TGF-β–mediated changes in keratin expression and were not appreciable following short exposure to these factors.

Biochemical effects of TGF-β2 on human raft cultures are dominant over those caused by RA

Since TGF-βs enhance the type of differentiation typically associated with wound-healing, whereas retinoids inhibit this program, we wondered whether one of the two extracellular factors might be dominant over the other. Previously, it had been reported that 1×10^{-6} M RA can prompt mouse keratinocytes to produce and secrete ~20 pM active TGF-β2 (Glick *et al.,* 1989). If human epidermal raft cells behaved in a similar fashion, then we would have expected that the combined effects of retinoic acid and 4–40 pM TGF-β2 might be similar to those exhibited by retinoic acid alone. To test this possibility, we cultured epidermal rafts in the presence of both RA and TGF-β2 and examined keratin biosynthesis (Figure 5C). Rafts cultivated for 3 wk in the presence of 1×10^{-7} M RA and 40 pM TGF-β2 showed depression of K1 and K13 synthesis and elevation of K16 synthesis (cf. lane 1 in A, untreated raft cultures, with lane 2 in C, with cultures 40 pM TGF-β2/1×10^{-7} M RA). At 400 pM TGF-β2 and 1×10^{-7} M RA, the pattern of K1, K13, and K16 biosynthesis was indistinguishable from that obtained with TGF-β2 alone (cf. lane 3 in C with lane 2 in A). Even when the RA concentration was raised to 1×10^{-6} M, the effects of TGF-β2 were dominant (Figure 5C, lanes 5–8). Moreover, the dominant effects of TGF-β2 could be elicited within 2 d, even when cultures had been grown for 3 wk in the presence of 1×10^{-7} or 1×10^{-6} M RA (Figure 5C, lanes 4 and 8, respectively).

Immunoblot analyses of proteins generated from raft cultures treated with 40–400 pM TGF-β2 and/or 1×10^{-7} to 1×10^{-6} M RA were consistent with our biosynthesis studies (Figure 6), with one exception: the absolute relative levels of K13 differed from the relative levels of their biosynthetic rates (cf. Figure 5 data with Figure 6, anti-hK13). Hence, although the K13 synthesis rate was low in raft cultures (Figure 5B, lane 1), the absolute level was quite high (Figure 6, lane 1). Most likely this occurred because K13 accumulates in the metabolically inactive s.c. layers, while K13 synthesis is in the spinous cell

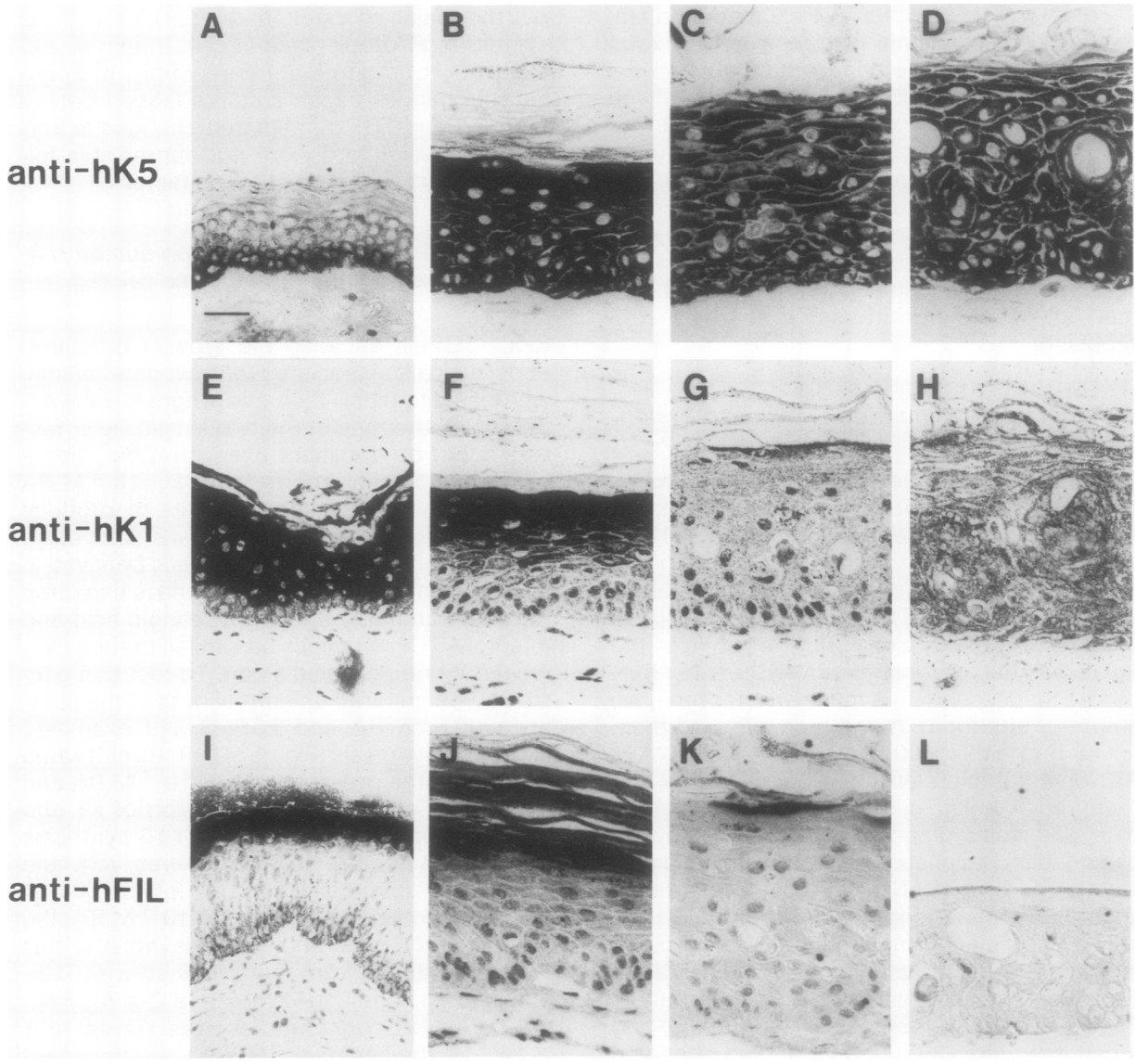


Figure 4. **Immunohistochemistry of epidermal rafts cultured in the presence of TGF-β2.** After 3 wk culture at the air–liquid interface, epidermal rafts were fixed in Carnoy's solution, embedded in paraffin, and sectioned (5 μm). For controls, foreskin was also fixed and embedded in the same way. Sections were deparaffinized, hydrated, and stained with each primary antibody. The primary antibody was detected with gold conjugated secondary antibody and silver enhancement. All sections for each antibody were developed for the same time. Sections were taken from foreskin (frames A, E, I, M, Q, and U), rafts cultured in the presence of 0 pM TGF-β2 (frames B, F, J, N, R, and V), rafts cultured with 40 pM TGF-β2 (frames C, G, K, O, S, and W), and rafts cultured with 400 pM TGF-β2 (frames D, H, L, P, T, and X). Antibodies used for immunostaining are given at the left of each set of sections. Note: apparent basal staining in frames E, I, M, Q, and U is actually due to the presence of melanin granules in the basal layer, rather than antibody staining. Bar = 27.5 μm.

(Kopan *et al.*, 1987). Upon RA treatment, K13 synthesis was elevated, but s.c. formation was inhibited, and thus, overall K13 levels were not greatly affected. In contrast, since TGF-β2 inhibits both s.c. formation and K13 synthesis, the differences between TGF-β–treated and untreated cultures were even greater when total protein levels were compared with biosynthetic rates.

RA does not induce appreciable expression of TGF-β2 mRNAs in human epidermal cultures

Our biochemical studies indicated that the effects of retinoids on raft cultures could not be explained by RA-mediated induction of TGF-β2. Moreover, since the effects of 40 pM TGF-β2 on keratin expression were dominant over those

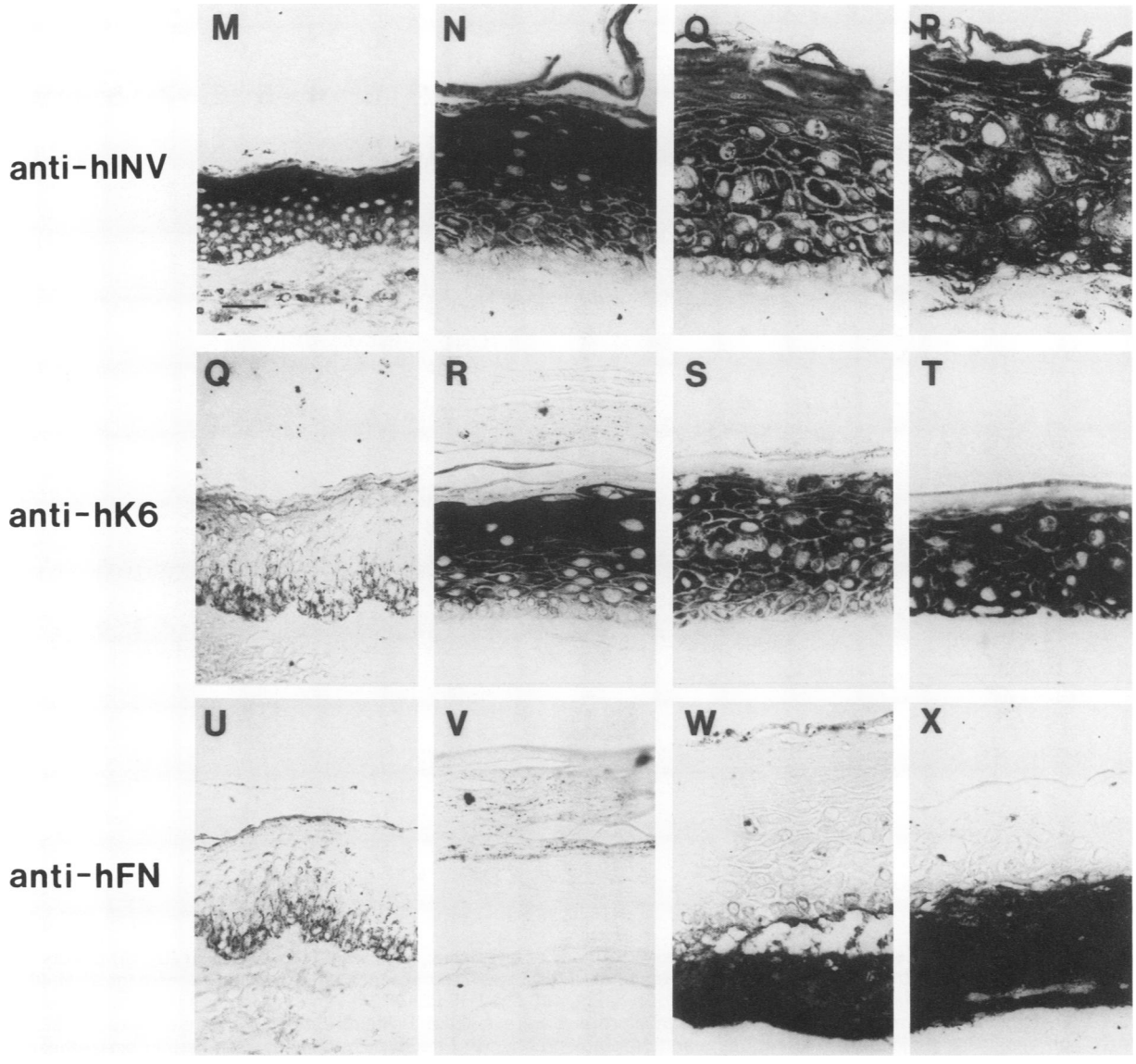


Figure 4. **(Continued)**

elicited by RA, it seemed unlikely that 20 pM active TGF-β2 was produced by the human raft cultures, as was seen in mouse keratinocytes grown submerged in culture medium (Glick *et al.,* 1989). That there may be a real and meaningful difference between the human raft and mouse keratinocyte cultures with respect to RA-mediated TGF-β2 expression was further suggested by the fact that 1×10^{-7} M RA accelerated proliferation in raft cultures (Kopan and Fuchs, 1989) and inhibited proliferation in submerged mouse keratinocyte cultures (Glick *et al.,* 1989).

To further explore the possibility that human and mouse keratinocytes behave differently under their respective culture conditions, we measured relative levels of TGF-β2 mRNA and control K14 mRNA in human epidermal cells cultured on feeder layers in the absence or presence of 1×10^{-6} M RA. Although the need for higher cell numbers precluded the use of raft cultures for this experiment, previous studies have shown that raft and feeder cultures are similar in their response to TGF-βs and RA with regards to proliferation and/or keratin expression (Fuchs and Green, 1981; Kopan *et al.,* 1987; Mansbridge and Hanawalt, 1988). Northern blot analysis revealed a hybridizing band from 1 μg poly A^+ RNA probed with digoxigenin-UTP–labeled K14 probe (Figure 7A, 1.6-kb band). In

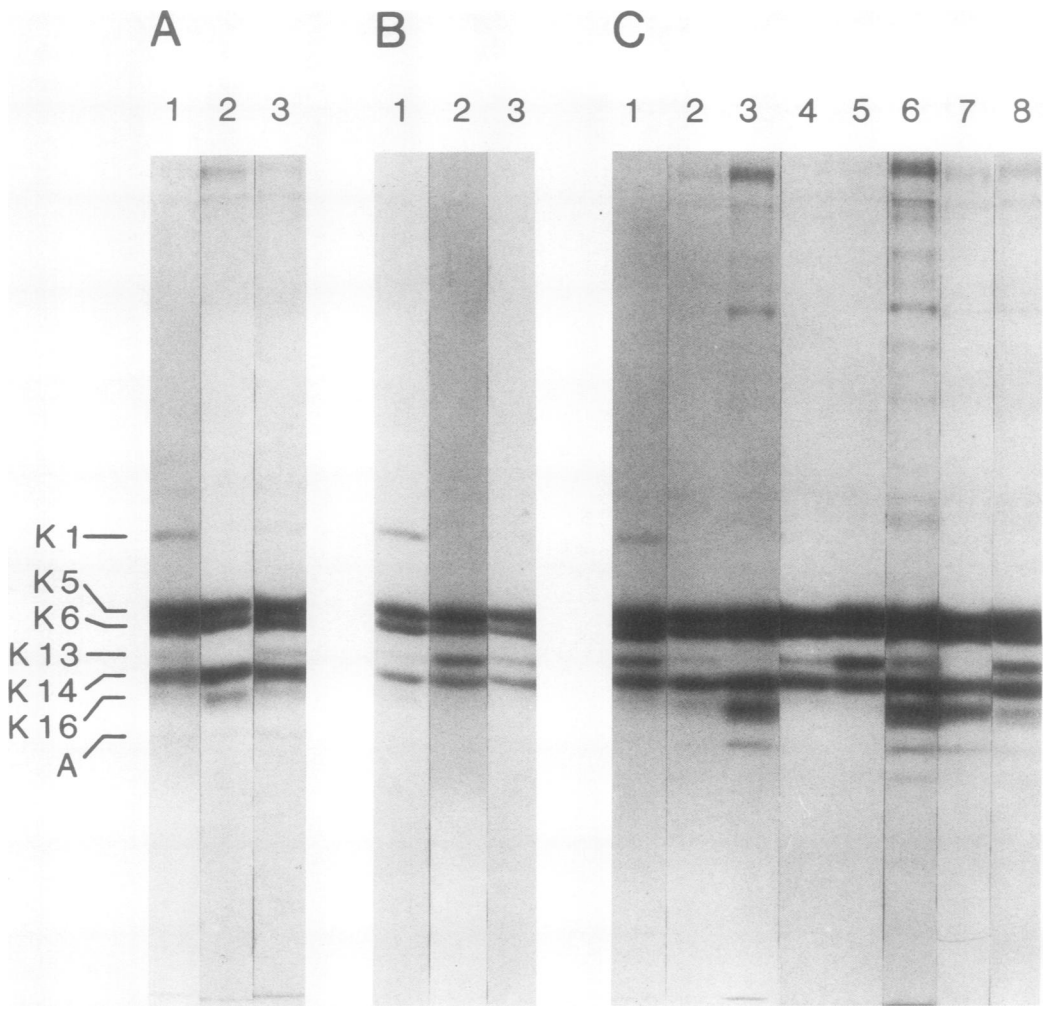


Figure 5. **[^{35}S]-methionine labeling of keratins after treatment of epidermal rafts with TGF-β2 and/or RA.** (A) Effects of TGF-β2 on keratin biosynthesis. Epidermal rafts were cultured for 3 wk in the absence (lane 1) or presence (lane 2) of 400 pM TGF-β2, or in the absence of TGF-β2 for 18 d, followed by 400 pM TGF-β2 for 2 d (lane 3). Cultures were then labeled with [^{35}S]-methionine overnight, and the triton-insoluble proteins were isolated. Proteins (5 μg each) were analyzed by SDS-PAGE and gels were subjected to fluorography and autoradiography. (B) Effects of retinoic acid on keratin biosynthesis. Epidermal rafts were cultured for 3 wk in the absence (lane 1) or presence (lane 2) of 10^{-6} M RA, or in the absence of RA for 18 d, followed by treatment with 10^{-6} M RA for 2 d (lane 3). Protein extraction and analyses were conducted as in A. (C) Effects of both RA and TGF-β2 on keratin biosynthesis. Rafts were cultured for 3 wk in the presence of 10^{-7} M RA (lane 1); 10^{-7} M RA + 40 pM TGF-β2 (lane 2); 10^{-7} M RA + 400 pM TGF-β2 (lane 3); 10^{-7} M RA for 18 d, followed by 10^{-7} M RA + 400 pM TGF-β2 for 2 d (lane 4); 10^{-6} M RA (lane 5); 10^{-6} M RA + 40 pM TGF-β2 (lane 6); 10^{-6} M RA + 400 pM TGF-β2 (lane 7); and 10^{-6} M RA for 18 d, followed by 10^{-6} M RA + 400 pM TGF-β2 for 2 d (lane 8). Protein extractions and analyses were as in A. Note: high molecular mass bands in lanes 3 and 6 represent contamination of triton-soluble proteins in the two samples. These bands were not consistently seen.

contrast, even when four times as much poly A$^+$ RNA was used with 20× longer developing times, we were unable to detect TGF-β2 mRNA with a comparable labeled probe specific for this message (Figure 7A, 6.5 kb; 4.1 kb). Our failure to detect TGF-β2 mRNA could not be attributed to the quality of the probe, since this probe could readily detect <1 pg of homologous TGF-β2 DNA (Figure 7B). Thus, if present, TGF-β2 mRNAs in human epidermal cells are likely to be rare (<1 in 4×10^6 poly A$^+$ RNAs), and this level does not seem to be greatly elevated as a consequence of RA treatment under conditions where RA acts as a mitogen. Although we cannot exclude the possibility that TGF-β2 induction by RA may occur at early time periods and may be transient, thus escaping our detection, all of our prior results were consistent with our failure to detect RA-mediated induction of TGF-β2 mRNA. Whether the differences in the behavior

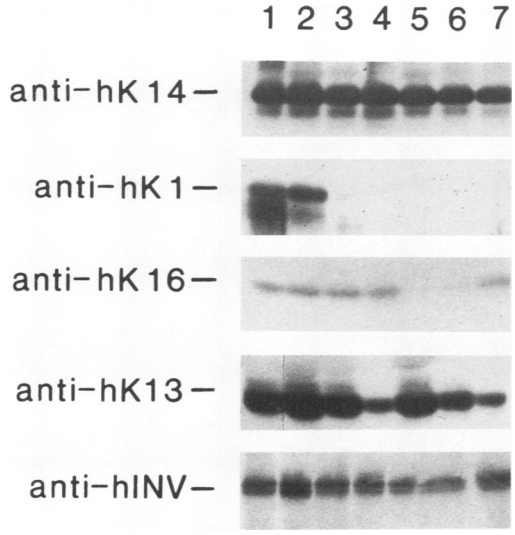


Figure 6. **Immunoblot analysis of proteins from epidermal rafts treated with both TGF-β2 and RA.** Rafts were cultured for 3 wk in the absence of RA and TGF-β2 (lane 1), or in the presence of 10^{-7} M RA (lane 2), 10^{-7} M RA + 40 pM TGF-β2 (lane 3), 10^{-7} M RA + 400 pM TGF-β2 (lane 4), 10^{-6} M RA (lane 5), 10^{-6} M RA + 40 pM TGF-β2 (lane 6), and 10^{-6} M RA + 400 pM TGF-β2 (lane 7). Triton-insoluble proteins were extracted and for each set of conditions, equal amounts of proteins were subjected to SDS-PAGE and immunoblot analysis. Antibodies used are indicated at left. Sample sizes varied for different antibodies, according to the strength of the antiserum (anti-hK14, 0.2 μg per well; anti-hK1, anti-hK13, anti-hK16, and anti-hINV, 5 μg per well).

of mouse and human keratinocytes to RA is a reflection of species-specific differences, the presence of raft fibroblasts, culturing cells at the air–liquid interface, or some other component of the culture system, remains to be determined.

TGF-β2 affects the differentiation program of the squamous cell carcinoma cell line SCC-13, but its effects are less prominent than on normal epidermal cells

SCC-13 cells are an immortalized epidermal cell line derived from a squamous cell carcinoma of the skin (Wu and Rheinwald, 1981). When cultured on collagen/fibroblast lattices, these cells express higher levels of K6 and K16 than normal epidermal raft cultures (Kopan and Fuchs, 1989). Moreover, SCC-13 raft cultures show a marked elevation in proliferative capacity when compared with normal epidermal rafts (Kopan and Fuchs, 1989). To determine whether TGF-βs could affect SCC-13 cultures in a fashion analogous to its action on normal epidermal rafts, we repeated all of our studies using SCC-13 cultures as a model system. In most cases, TGF-β2 elicited effects on SCC-13 cultures that were similar to those achieved with normal cultures. Thus, e.g., as judged by immunoblot analysis, expression of K1, K13 and filaggrin were inhibited, whereas K16 expression was enhanced (data not shown). TGF-β2 also markedly inhibited DNA synthesis, but the inhibition was not as complete as it was in normal epidermal cells. Thus, at 4 pM, TGF-β2 showed only a slight inhibition of [^{3}H]-thymidine incorporation (cf. Figure 8A, no TGF-β2 to 8B, 4 pM TGF-β2). At 40 pM, TGF-β2 showed appreciable inhibition of labeling in the basal layer (Figure

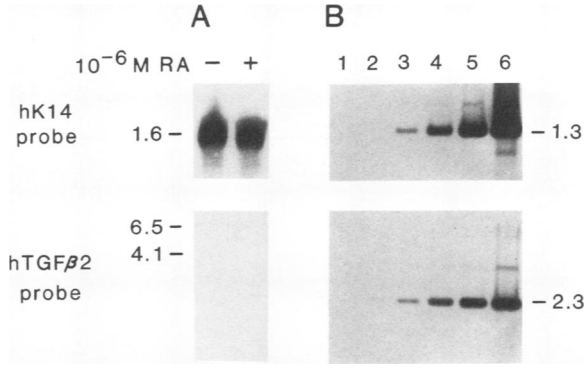
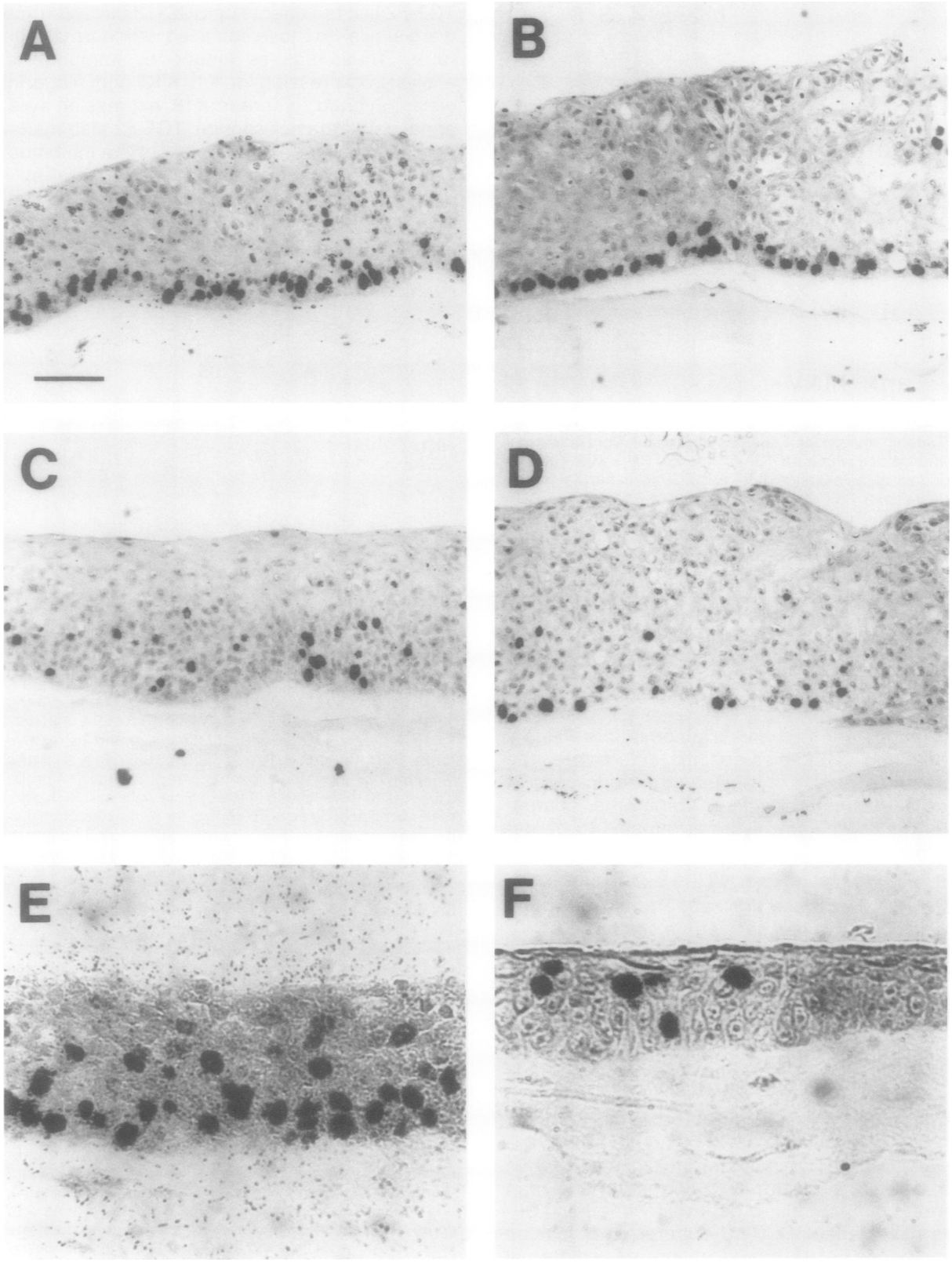


Figure 7. **Effects of RA on expression of TGF-β2 mRNA.** Human epidermal cells were cultured on plastic in the presence of a fibroblast feeder layer. Cells were cultured in the absence (7A; −) or presence (7A; +) of 10^{-6} M RA, and poly A$^+$ RNAs were isolated after EDTA treatment to remove feeder cells (Chomczynski and Sacchi, 1987). Poly A$^+$ RNAs (4 μg for TGF-β2 probe experiment and 1 μg for K14 probe experiment) were loaded in the sample wells of 0.85% agarose-formaldehyde gels. To control for probe detection limits, serial dilutions of TGF-β2 cDNA (pPC21; 2.3-kb insert; Madisen *et al.*, 1988) and K14 cDNA (3sp; 1.3 kb; Vassar *et al.*, 1989) were also loaded (7B). Control DNAs for K14 were 12× the amount of TGF-β DNAs, since subsequent exposure times were 12× less for K14 than TGF-β2 blots. Values for TGF-β DNAs are 0, lane 1; 1 pg, lane 2; 10 pg, lane 3; 100 pg, lane 4; 1 ng, lane 5; and 10 ng, lane 6. After electrophoresis, RNAs and DNAs were transferred to nitrocellulose by blotting (Thomas, 1980). For riboprobe generation, the TGF-β2 insert from pPC21, containing the full-length coding and 3′ untranslated sequence was subcloned in the 3′ to 5′ direction into the *Eco* RI site of the RNA expression vector pSG5 (obtained from Dr. Pierre Chambon, Strasburg, France). Complementary TGF-β2 RNA was prepared by linearizing the clone with *Bam* HI and using T7 RNA polymerase for transcription. Complementary K14 cRNA was prepared as described (Vassar *et al.*, 1989). Digoxigenin-labeled cRNA riboprobes specific for TGF-β2 and K14 mRNAs were prepared using digoxigenin-UTP as described by the manufacturer (Boehringer-Mannheim Biochemicals, Indianapolis, IN), and equal amounts of labeled TGF-β2 and K14 cRNAs with comparable specific activities were hybridized to duplicate blots. RNAs were detected by goat antisera against digoxigenin. For K14, color was developed for 15 min. For TGF-β2, color development was continued for 3 h. The numbers at left indicate expected sizes for K14 and TGF β2 mRNAs.


A
B
C
D
E
F

8C), but even at 400 pM TGF-β2, some basal SCC-13 cells still incorporated radiolabel (Figure 8D). Interestingly, a few suprabasal SCC-13 cells also showed [^{3}H]-thymidine labeling in cultures treated with 0–400 pM TGF-β2, and these cells seemed to be less sensitive to TGF-β2 than cells in the innermost, basal layers. These findings were different from those obtained with normal epidermal rafts, where no suprabasal labeling with [^{3}H]-thymidine was detected, and where a near complete block in DNA synthesis was achieved with 400 pM TGF-β2.

That SCC-13 cultures are less sensitive to TGF-β2 was also suggested by morphological studies. Hence, the number of vacuoles and pyknotic nuclei increased somewhat in the presence of 40–400 pM TGF-β2 (Figure 8, C and D), but overall, the morphological differences were not as great as those elicited by TGF-β2 treatment of normal epidermal cells. It seems likely that the lack of appreciable TGF-β2–induced morphological changes in SCC-13 cultures is due to the fact that many of the phenotypic features associated with TGF-β2-treated cultures were already present in SCC-13 cultures. In summary, our results on SCC-13 cultures emphasized the uncoupling of SCC-13-like epidermal differentiation and cell proliferation: despite the fact that TGF-β2 promoted withdrawal of these cells from the cell cycle, it enhanced slightly the biochemical phenotype typical of untreated SCC-13 cultures that are undergoing hyperproliferation.

TGF-βs are dominant over retinoids in their ability to alter proliferation of human SCC-13 raft cultures

Previously, we had shown that SCC-13 raft cultures are more sensitive to retinoids than normal raft cultures, resulting in loss of most features of epidermal differentiation at 1×10^{-7} M RA (Kopan and Fuchs, 1989). Since proliferation of RA-treated SCC-13 cells is much higher than normal RA-treated cultures (Kopan and Fuchs, 1989), the tumor cells provided a good model for examining whether TGF-βs or RA were dominant with respect to their ability to influence proliferation of basal keratinocytes. To examine the combined effects of RA and TGF-βs on SCC-13 keratinocyte proliferation, we cultured SCC-13 keratinocyte rafts for 10 d in the presence of 1×10^{-7} M RA, ±400 pM TGF-β2. Figure 8, E and F, shows representative sections of these cultures after [^{3}H]-thymidine labeling, fixation, and autoradiography. Similar to the dominant effects of TGF-βs over RA on the biochemistry of differentiating epidermal cells, TGF-βs were dominant in their inhibitory action on proliferation of basal keratinocytes.

Discussion

TGF-βs as regulators of growth

TGF-βs have a profound inhibitory effect on keratinocyte growth (Shipley *et al.*, 1986; Kopan *et al.*, 1987; Coffey *et al.*, 1988). Using human epidermal raft cultures, we confirmed earlier reports that these effects are largely reversible within a 3-d period (Shipley *et al.*, 1986). Our results have shown that as little as 4 pM TGF-β1 or TGF-β2 added to the serum-containing medium retarded human keratinocyte growth on raft cultures. However, 3-wk growth in 400 pM TGF-βs was necessary to completely block growth of normal epidermal raft cultures, and even at this high level, TGF-βs did not completely inhibit growth in rafts of the squamous cell carcinoma line, SCC-13. In contrast to short-term exposure, prolonged growth of normal rafts in 400 pM TGF-β2 was largely but not fully irreversible. This finding was consistent with our detection of biochemical markers of terminal differentiation in many of the basal cells of these cultures.

The appearance of terminally differentiating cells in the basal layer of cultures treated with high levels of TGF-βs for a prolonged time suggests the possibility that unusually extended periods of cell cycle withdrawal might lead to terminal differentiation even in basal cells. However, the significant lag time between cessation of cell growth and irreversible commitment to terminal differentiation indicates the likelihood that a second, as yet unidentified, molecular event follows TGF-β-induced withdrawal from the cell cycle. This additional step seems to be necessary for progression of a nondividing cell in a reversible state to a non-

Figure 8. **[^{3}H]-thymidine labeling of SCC-13 cells treated with TGF-β2, RA, and TGF-β2 + RA.** SCC-13 cells were cultured at the air–liquid interface in the presence of 0 pM TGF-β2 (A), 4 pM TGF-β2 (B), 40 pM TGF-β2 (C), 400 pM TGF-β2 (D), 1×10^{-7} M RA (E), or 1×10^{-7} M RA + 400 pM TGF-β2 (F). Rafts were labeled with [^{3}H]-thymidine for 2 h before harvesting, fixed in Carnoy's solution, and embedded in paraffin. Sections (5 μm) were deparaffinized, dehydrated, and exposed to Kodak NTB2 emulsion for 3 wk. Sections were developed and then stained with hematoxylin and eosin. Black grains indicate nuclear labeling. Bar = 68.8 μm (A–D); bar = 27.5 μm (E and F).

dividing cell irreversibly committed to terminal differentiation.

TGF-βs and their biochemical and morphological effects on differentiation

Our results confirm and extend previous studies by Mansbridge and Hanawalt (1988), demonstrating that TGF-βs can have profound effects on epidermal differentiation as well as cell growth. Surprisingly, the TGF-β–mediated effects on terminal differentiation were ones characteristic of hyperproliferating, rather than hypoproliferating, epidermis. A priori, it seems unusual that a factor which can cause marked inhibition of epidermal cell growth can simultaneously elicit morphological and biochemical changes more frequently associated with hyperproliferative diseases of the skin. However, this phenomenon could arise if 1) the abnormal keratinization biochemistry is independent of the proliferative state of the basal layer, as suggested previously (Kopan and Fuchs, 1989; Schermer *et al.,* 1989), and if 2) positive acting growth factors can sometimes be dominant over negative ones. In this regard, even though TGF-α and TGF-βs have opposite effects on epidermal proliferation, they are both present, e.g., during wound-healing when the epidermis undergoes hyperproliferation and the abnormal biochemistry of keratinization is observed (Mansbridge and Knapp, 1987). Levels of TGF-α and TGF-β mRNAs are also elevated by tumor-promoting agent, which promotes both keratinization and tumorigenesis when topically applied to mouse skin (Akhurst *et al.,* 1988; Pittlekow *et al.,* 1989). Hence, it seems likely that it is TGF-α which plays a major role in hyperproliferation and TGF-βs which play a major role in the biochemical effects on differentiation commonly associated with hyperproliferation. Whether other inhibitors of keratinocyte growth, e.g., epidermal chalones, influence the biochemistry of epidermal differentiation in a fashion similar to TGF-βs remains to be determined. Such knowledge may be useful in understanding the functional significance of these changes in keratin gene expression.

The fact that K6/K16 expression is typically only transiently expressed during wound-healing when TGF-β levels are unusually high suggests that the routine role of lower levels of TGF-βs in epidermal cells is to control growth, rather than to modify differentiation. Moreover, since the dose responses appear to be different for the two effects, it suggests the possibility that the effect of TGF-βs on differentiating keratinocytes may be at least in part distinct from the effects of TGF-βs on dividing keratinocytes.

We do not know whether the effects that we have observed on epidermal differentiation are a direct or indirect consequence of the presence of TGF-βs. We have made no attempt to use serum-free medium, and hence we cannot rule out the possibility that the effects of TGF-βs are potentiated via some other growth factor present in the serum. Similarly, we have not examined whether the effects occur in the absence of fibroblasts that are otherwise present in our collagen lattices. In this regard, we did find that at least one biochemical change, namely fibronectin expression, is enhanced greatly in the lattice fibroblasts as a consequence of TGF-β addition (see also Ignotz and Massague, 1987). Although direct contact of keratinocytes with fibronectin inhibits terminal differentiation (Adams and Watt, 1989), the TGF-β–mediated induction of fibronectin seemed to be confined to the fibroblasts in the dermal lattice, and hence it is unlikely that elevated fibronectin levels in the dermal lattice was responsible for the changes in keratinocyte differentiation that we observed. However, additional studies will be necessary to determine the molecular mechanisms underlying the effects of TGF-βs on epidermal cell growth and differentiation.

Can TGF-βs and retinoic acid act on epidermal cells that have undergone commitment to terminally differentiate?

Since retinoids play a major role in human keratinocyte growth and cell migration (Fuchs and Green, 1981) and since TGF-βs act to inhibit keratinocyte proliferation, it is clear that retinoids and TGF-βs can act on the basal cell population. The stage of epidermal differentiation at which retinoids and TGF-βs act to control filaggrin and keratin gene expression is less clear. It could be that these factors act only on basal cells and that they alter the differentiative program before commitment of a cell to keratinize. In this case, the factors would act indirectly to control the program of gene expression in differentiating cells. Alternatively, retinoids and TGF-βs might act on suprabasal cells after they have undergone a commitment to terminally differentiate. In this case, the action of the factors on filaggrin, K6/K16 and K1/K10 could either be direct or indirect.

The notion that retinoids and TGF-βs might act on suprabasal cells is consistent with an hypothesis by Galvin *et al.* (1989) proposing that an epidermal cell may be able to tailor its dif-

ferentiative program after commitment has taken place. In support of this notion are recent studies by Noji *et al.* (1989) indicating that the retinoic acid receptor mRNAs are most prevalent in the keratinizing cells of the skin. Receptors for TGF-β have not yet been characterized in the skin, but it seems likely that they too might be found in all cell layers that are still metabolically active. If TGF-β can act on keratinizing cells independently of their action on basal cells, this would explain why the morphological and biochemical features in the suprabasal layers of TGF-β–treated cultures are also exhibited in keratinizing layers of hyperproliferating epidermis. Despite the attractiveness of the hypothesis that retinoids and TGF-βs can act after a cell has undergone a commitment to terminally differentiate, the relatively rapid rate of upward cell migration in 3 wk raft cultures (Choi and Fuchs, unpublished data) precluded our ability to unequivocally resolve this issue.

We do not yet know the molecular mechanisms that underly the effects of TGF-βs and retinoids on keratin and filaggrin expression. A search for TGF-β1 inhibitory elements (Kerr *et al.,* 1990) in the 5′ upstream sequences of the human genes encoding K1 (Johnson *et al.,* 1985) and K10 (Rieger and Franke, 1988) did not reveal any sequences with 100% identity to the putative consensus sequence. Similarly, we did not find any sequence with 100% identity to the putative retinoic acid response element (de The *et al.,* 1990) in the 5′ upstream sequences of the human genes encoding K1 (Johnson *et al.,* 1985), K6b (Tyner *et al.,* 1985), and K16 (Rosenberg *et al.,* 1988). As further studies are conducted, we should be able to determine whether these changes in keratin expression are transcriptional, and if so, whether they are directly or indirectly mediated via the type of response elements identified by other researchers for nonkeratinocyte genes.

Some antagonistic effects of TGF-βs are dominant over those of RA

The dominant behavior of TGF-βs over RA in our human epidermal raft cultures encompassed both keratin expression and cell proliferation and was observed in both normal and malignant keratinocytes. At first glance, this might be seemingly in contrast to the results of Glick *et al.* (1989), who showed that mouse keratinocytes produce and secrete >20 pM active TGF-β2 in response to 1×10^{-6} M RA. However, proliferation of RA-treated mouse keratinocytes is also inhibited, whereas human raft cultures treated with 1×10^{-6} M RA respond by enhancing proliferation. Coupled with our failure to detect TGF-β2 mRNA in RA-treated cultures, our results are consistent with those of Glick *et al.* (1989), and furthermore suggest that in mouse keratinocytes as in human keratinocytes, the effects of TGF-βs are dominant over those of retinoids. Whether the effects of TGF-βs are always dominant over those of RA in keratinocytes remains to be investigated.

The fact that human and mouse keratinocytes behave differently in their response to retinoids is not surprising. Species specific differences in the way keratinocytes respond to a particular concentration of an extracellular regulator have been described before, particularly with respect to calcium-induced keratinization, which readily occurs in mouse keratinocytes under conditions where human keratinocytes are largely unaffected (Rheinwald and Green, 1975; Hennings *et al.,* 1980; Watt *et al.,* 1984). In fact, one major difference in the culture media used in the two sets of experiments is that the mouse keratinocytes studied by Glick *et al.* (1989) were grown in the presence of 0.05 mM Ca^{++}, whereas our human keratinocytes were cultured on medium containing 1.3 mM Ca^{++}. Whether these differences in cell culture conditions or species can account for the differences in RA action on mouse and human keratinocytes remains to be shown.

Conclusions

Table 1 summarizes our results. Our experiments have shown that TGF-βs and retinoids have profound and pleiotropic effects on human raft cultures. In some regards, e.g., suppression of K1 and filaggrin expression, the action of TGF-βs and RA can be synergistic. In other regards, e.g., proliferation and K6/K16 expression, the action of TGF-βs can be both antagonistic and dominant over RA. Under the conditions used here, RA did not seem to induce TGF-β expression in human raft cultures. The differences between our findings with human raft cultures and those of Glick *et al.* (1989) with mouse keratinocytes, reveal the existence of an intriguing and complex interrelation between TGF-βs and RA that in some cases may be uncoupled. This may explain why retinoids sometimes enhance and othertimes inhibit proliferation in keratinocyte populations.

The effects of TGF-βs seem to be less variable than those of retinoids, and are usually reversible, although prolonged incubation of cells in high concentrations of TGF-βs can lead to

Table 1. Summary of the effects of TGF-β and RA on proliferation, morphology, and keratinization of human raft cultures

	Normal epidermal							SCC-13						
	TGF-β				RA†		TGF-β + RA‡	TGF-β			RA		TGF-β + RA
	0 pM	4 pM	40 pM	400 pM	−7 M	−6 M		0 pM	4 pM	40 pM	400 pM	−7 M	−6 M	
Basal cell proliferation	+++	++	+	+/−	++++	+++	+/−	++++++	+++++	++++	++	++++++++	++++++++	++
S. corneum	++++++	+++++	++	+/−	++++	+/−	−	+/−	−	−	−	−	−	−
Granular layer	++	+/−	−	−	+/−	−	−	++§	−	−	−	−	−	−
Keratin pearls	−	−	++	+++	−	−	+	+++	+++	++++	++++	−	−	+
K6/K16	++++	++++	+++++	+++++	++++	+++	++++	++++	+++++	+++++	+++++	++	+/−	+++
K1/K10	+++	++	+/−	+/−	++	+/−	−	++++	++	+/−	+/−	−	−	−
Filaggrin	+++	+/−	+/−	−	+/−	−	−	+++	+++	+	−	n.d.	n.d.	n.d.
K13	++++	+++	++	+	+++++	+++++	+	+++	++	−	−	++	+	−
Involucrin	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	n.d.	n.d.	n.d.
K14/K5	+++++	++++++	+++++++	+++++++	+++++	++++	+++++	++++	++++	+++++	+++++	+++	+	++

n.d., not determined.
† RA values are 10^{-7} M (−7 M) and 10^{-6} M (−6 M).
‡ TGF-β + RA values are 400 pM TGF-β and 10^{-6} M RA.
§ Granular cells are not confined to a discrete layer in SCC-13 cultures.

largely irreversible terminal differentiation. Differentiating keratinocytes make their own low levels of TGF-β mRNAs, and their suprabasal location suggests that autocrine TGF-β may be involved in maintaining cessation of cell growth in keratinizing layers. At higher levels, TGF-βs suppress some features of keratinization and enhance features more commonly associated with epidermal hyperproliferation. TGF-βs can also inhibit growth of basal cells, but their action on basal and keratinizing cells may be separable, as judged by our findings that some TGF-β–mediated effects on keratinizing cells can be uncoupled from the proliferative state of the basal keratinocyte. As additional experiments are conducted, the precise events leading to the interrelation between TGF-βs, retinoic acid, and the control of epidermal growth and differentiation should become more clear.

Materials and methods

Preparation of raft cultures

Collagen/fibroblast lattices were prepared as described previously (Asselineau *et al.*, 1985), and seeded with either 1) human epidermal cells derived from foreskin tissue and cloned from cultures grown on plastic in the presence of fibroblast feeder cells (see Kopan *et al.*, 1987 for details), or 2) human SCC-13 cells derived from a squamous cell carcinoma of the skin (Wu and Rheinwald, 1981). Cells were grown submerged in medium for 7 d, and then floated for 3 wk (normal rafts) or 10 d (SCC-13 rafts) on stainless steel grids. TGF-β2 and TGF-β1 (R & D Systems, Minneapolis, MN) was added to 4–400 pM wherever indicated, and medium was changed every 2 d. TGF-βs were stored at −70°C as 100× stocks in 4 mM HCl and 0.1 mg/ml bovine serum albumin (BSA). Control cultures for TGF-β experiments received a 1:100 dilution of 4 mM HCl, 0.1 mg/ml BSA. RA (Sigma Chemical Co., St. Louis, MO) was added at either 1 $\times$ 10^{-7} or 1 $\times$ 10^{-6} M wherever indicated in the text. RA was stored in light-proof containers at −70°C as 4000× stocks in dimethyl sulfoxide (DMSO). Control cultures for RA experiments received a 1:4000 dilution of DMSO.

[^{3}H]-Thymidine labeling, fixation, autoradiography and hematoxylin/eosin staining of raft cultures

Three-week raft cultures were fed with fresh medium, and 12 h later, cultures were labeled with 2 μCi/ml of [^{3}H]-thymidine (84 Ci/mmol, Amersham Corp., Arlington Heights, IL) for 2 h as described (Kopan and Fuchs, 1989). After extensive washing with PBS, cultures were fixed in Carnoy's (6:3:1 ratio of ethanol:chloroform:acetic acid, respectively) for 30 min. Fixed cultures were embedded in paraffin and sectioned (5 μm). For autoradiography, sections were deparaffinized, exposed to Kodak NTB2 liquid nuclear track emulsion (Eastman Kodak, Rochester, NY) for 3 wk, developed, and stained with hematoxylin and eosin.

Immunohistochemistry

Fixed, paraffin-embedded sections (5 μm) were hydrated prior to immunohistochemical staining. Staining was carried

out as described by Kopan and Fuchs (1989). Antisera and dilutions used were as follows: rabbit polyclonal antisera anti-hK6 and anti-hK1, 1:100 (Stoler *et al.,* 1988); rabbit polyclonal antisera anti-hK5, 1:200 (Lersch and Fuchs, unpublished results); rabbit polyclonal anti-human fibronectin antisera (anti-hFN), 1:200 (Biomedical Technologies Inc., Stoughton, MA); rabbit polyclonal antisera against human involucrin (anti-INV), 1:20 (Biomedical Technologies, Inc.); and mouse monoclonal antibody against human filaggrin (anti-hFIL), 1:200 (Biomedical Technologies, Inc.). For anti-hFN staining, 5% BSA in phosphate-buffered saline (PBS) was used instead of 20% normal goat serum since normal serum gives a high background staining because of the presence of fibronectin in the serum. Following incubation with primary antisera, slides were subjected to immunogold enhancement as described by Kopan and Fuchs (1989).

Immunoblot analysis

Triton soluble proteins from raft cultures were extracted with 1% Triton X-100 in 150 mM NaCl, 10 mM Tris tris(hydroxymethyl)aminomethane · HCl, pH 7.4, 2 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM iodoacetic acid, 1 mM benzamidine, and 10 μg/ml each of aprotinin, pepstatin, leupeptin, and antipain (Sigma). This fraction was used for immunoblot analysis of fibronectin. The triton-insoluble pellet was dispersed by sonication in a solution of 1% Triton X-100, 150 mM NaCl, and 10 mM Tris · HCl, pH 7.4, followed by centrifugation at 10 000 × *g.* After extensive washing, the pellet was dissolved in 8 M urea, 10% β-mercaptoethanol, and 2 mM PMSF. This fraction was used for immunoblot analysis of keratins, involucrin, and filaggrin. Protein concentrations were determined by the Biorad protein assay method (Biorad Laboratories, Richmond, CA). Equal amounts of proteins were separated by electrophoresis through SDS polyacrylamide gels (8.5%). Proteins were transferred to nitrocellulose paper by electroblotting, and total protein was visualized by staining the blot with 2% Ponceau S in 3% trichloroacetic acid to confirm that the protein loadings were approximately the same for each lane. The blots were then subjected to immunoblot analysis. The method used was essentially according to Towbin *et al.* (1979), except that 5% nonfat dry milk, 0.2% Triton X-100 in PBS was used as blocking buffer, primary antibody-binding buffer, and wash buffer. In addition to the antisera used for immunohistochemistry, we used a rabbit polyclonal anti-hK14 antisera (1:1000; Stoler *et al.,* 1988), a rabbit polyclonal anti-hK16 antisera (1:50; Rosenberg *et al.,* 1988), and a mouse monoclonal antibody (1C7), monospecific for K13, (1:10 dilution; van Muijen *et al.,* 1986). Each antibody was incubated for 1 h at room temperature and washed with three changes of buffer for 30 min. Primary antibody binding was detected with alkaline phosphate-coupled goat anti-rabbit immunoglobulin G (IgG) or goat antimouse IgG as necessary (Biorad Laboratories). NBT and BCIP were used as color developing reagents, according to the manufacturer's specifications (Biorad Laboratories).

Radiolabeling and extraction of proteins

Raft cultures were labeled overnight with 10 μCi/ml of [^{35}S]-methionine (1163 Ci/mmol, New England Nuclear Research Products, Boston, MA), and then washed 3× with PBS. Intermediate filament proteins were extracted and protein concentrations measured as described above. Proteins were resolved by electrophoresis through 8.5% polyacrylamide gels. For fluorography, gels were fixed, treated with 1 M sodium salicylate for 30 min, dried, and exposed to X-OMAT-AR film (Eastman Kodak Co., Rochester, NY).

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