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Transforming growth factor alpha induces collagen degradation and cell migration in differentiating human epidermal raft cultures

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When cultured on plastic and treated with transforming growth factor alpha (TGFα), human keratinocytes exhibit an increase in proliferation at the colony periphery, apparently as a consequence of enhanced cell migration (Barrandon and Green, 1987). To investigate the effects of TGFα on a differentiating stratified squamous epithelium and to begin to examine the molecular basis mediating this influence, we cultured human epidermal cells on a gelled lattice of collagen and fibroblasts, floating on the air-liquid interface. Under these conditions, raft cultures differentiate and exhibit morphological and biochemical features of human skin in vivo (Asselineau *et al.*, 1986; Kopan *et al.*, 1987). When 3-wk-old raft cultures were treated with TGFα, basal cells showed a marked increase in cell proliferation. At elevated concentrations of TGFα, the organization of cells within the artificial tissue changed and islands of basal cells entered the collagen matrix. Biochemical analysis of the response revealed that type I collagenase and gelatinase were induced by keratinocytes within 12 h after TGFα treatment. In contrast, invasion of basal cells into the collagen matrix was not significant until 48–72 h post-treatment, suggesting that collagenase and gelatinase production may be a prerequisite to this phenomenon. These results have important implications for the possible role of TGFα in squamous cell carcinoma and tumor invasion.

[1] Abbreviations used: EGF, epidermal growth factor; FN-R, fibronectin receptor; PCNA, proliferating cell nuclear antigen; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TGFα, transforming growth factor alpha; Tris, tris(hydroxymethyl)aminomethane.

Introduction

Transforming growth factor alpha (TGFα)[1] is a member of the epidermal growth factor (EGF) family. It shares functional and structural homology with EGF and it binds to the same cell-surface receptor (Massague, 1983). TGFα has been implicated in stimulating proliferation, migration, and angiogenesis in a variety of cell types during normal development, wound healing, and neoplasia (Derynck, 1990; Massague, 1990; Werb, 1990). In epidermis, TGFα seems to be a major autocrine growth factor. It is synthesized by cultured epidermal cells (Coffey *et al.*, 1987) and it has been localized to the epidermis of neonatal and adult skin biopsies (Gottlieb *et al.*, 1988). Some lines of evidence suggest that it is even more potent than EGF in stimulating growth of both normal keratinocytes (Rheinwald and Green, 1977; Barrandon and Green, 1987) and skin tumors (Gottlieb *et al.*, 1988; Finzi *et al.*, 1988). TGFα and EGF are also involved in epidermal repair and appear to act in concert to influence critical activities in inflammation and in wound-healing processes (for reviews see Wahl *et al.*, 1989; Lynch, 1991). In addition, elevated levels of TGFα have been reported in skin lesions of patients with psoriasis, a hyperproliferative disease of the skin (Gottlieb *et al.*, 1988; Elder *et al.*, 1989). Recently, transgenic mice studies using a keratin promoter to overexpress TGFα in skin have revealed possible roles for TGFα in epidermal thickening, hyperproliferation, papilloma formation, and psoriasis (Vassar and Fuchs, 1991).

Basal epidermal cells have EGF receptors on their surface, and a good correlation exists between receptor levels and TGFα/EGF responsiveness (Cohen and Elliott, 1963; Green *et al.*, 1983; Vassar and Fuchs, 1991). Thus it seems clear that the molecular mechanisms underlying TGFα-mediated effects on keratinocyte growth involve the classical signal transduction pathway mediated by these EGF receptors (Ullrich and Schlessinger, 1990; Oberg *et al.*, 1990). This said, the mechanism is likely to be more complex because a part of the growth response ap-

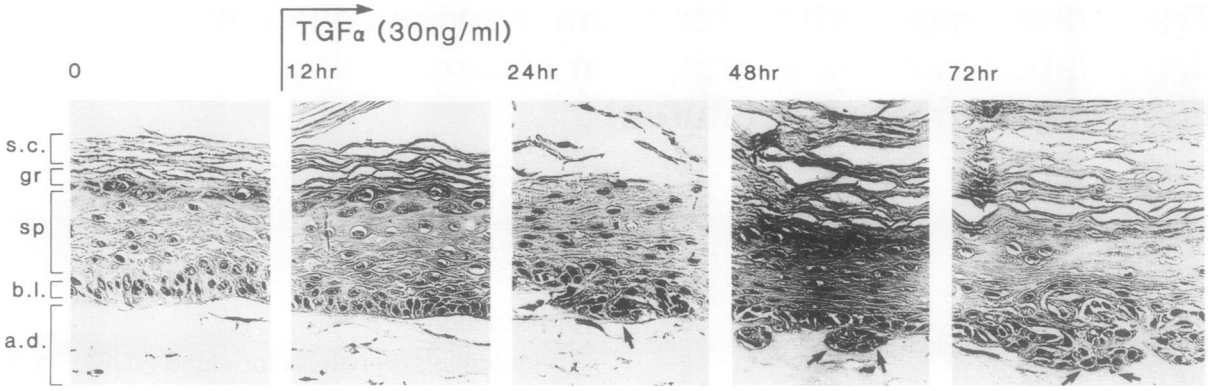

Figure 1. **Effects of TGFα on cell migration and stratified squamous epithelial morphology in human epidermal raft cultures.** Human epidermal keratinocytes were cultured on collagen/fibroblast lattices at the air liquid interface for 3 wk. TGFα (30 ng/ml) was then added to the culture medium, and at t = 0, 12, 24, 48, and 72 h thereafter, rafts were fixed in Carnoy's solution, embedded in paraffin, and sectioned (5μm). Sections were stained with hematoxylin and eosin and visualized by light microscopy. Arrows denote TGFα-treated, basal-like cells that have migrated into the artificial dermis. s.c., stratum corneum layers; gr, granular layers; sp, spinous layers; b.l., basal layer; a.d., artificial dermis (lattice of type I collagen and fibroblasts). (Magnification: ×155).

pears to be due to the ability of TGFα to enhance cell migration: when cultured on plastic, only large colonies of keratinocytes enhance their growth as a consequence of TGFα treatment, and this growth is almost exclusively at the colony periphery, where cell migration has also been enhanced (Barrandon and Green, 1987). It is not yet clear whether TGFα plays a role in cell migration in the context of a highly differentiated stratified squamous epithelium. In addition, the biochemical factors mediating this response have yet to be elucidated.

To investigate the effects of TGFα on the architecture and biochemistry of epidermal tissue, we have used an in vitro system whereby human epidermal cells are cultured on a floating lattice of collagen and fibroblasts. Under these conditions, human epidermal cells organize into a stratified squamous epithelium and display a high degree of morphological differentiation as well as terminal differentiation-specific gene expression (Asselineau *et al.,* 1986; Kopan *et al.,* 1987; Choi and Fuchs, 1990; for a review see Fuchs, 1990). Because this system is permissive for most of the differentiated properties of epidermis, it was possible to examine TGFα-mediated action at various stages of growth and differentiation. Using immunohistochemistry and [^{3}H]thymidine labeling, we explored the effects of TGFα and EGF on tissue morphology, cell proliferation, cell migration/invasion, and differentiation. Using [^{35}S]methionine labeling, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoprecipitation, and zymography, we investigated the effects of TGFα on collagenase and gelatinase production in keratinocytes. Our results have revealed that TGFα not only enhances basal cell proliferation but also causes the invasion of these cells into the artificial dermis. The TGFα-mediated changes in tissue architecture exhibit delayed kinetics and are preceded by proliferation, type I collagenase and gelatinase production, and collagen matrix degradation. These findings have important implications for the possible role of TGFα in skin tumorigenesis.

Results

TGFα and EGF cause an increase in cell migration/invasion and proliferation in the basal compartment of epidermal cultures

Human epidermal cells cultured in the absence of TGFα or EGF for 3 wk on a floating lattice of collagen and 3T3 fibroblasts stratified extensively and showed a gradual series of morphological changes characteristic of terminal differentiation (Figure 1) (Asselineau *et al.,* 1986; Kopan *et al.,* 1987). Most phases of normal morphology were preserved after 12 h of treatment with 30 ng/ml TGFα, a concentration known to enhance proliferation and cell migration in human keratinocytes cultured on plastic (Barrandon and Green, 1987). However, by 24 h post-TGFα treatment, some disorganization was evident in the innermost (basal) layer. Some basal cells left their normal position in the stratified squamous epithelium and invaded the collagen matrix. This movement was even more pronounced at 48–72 h after TGFα treatment,

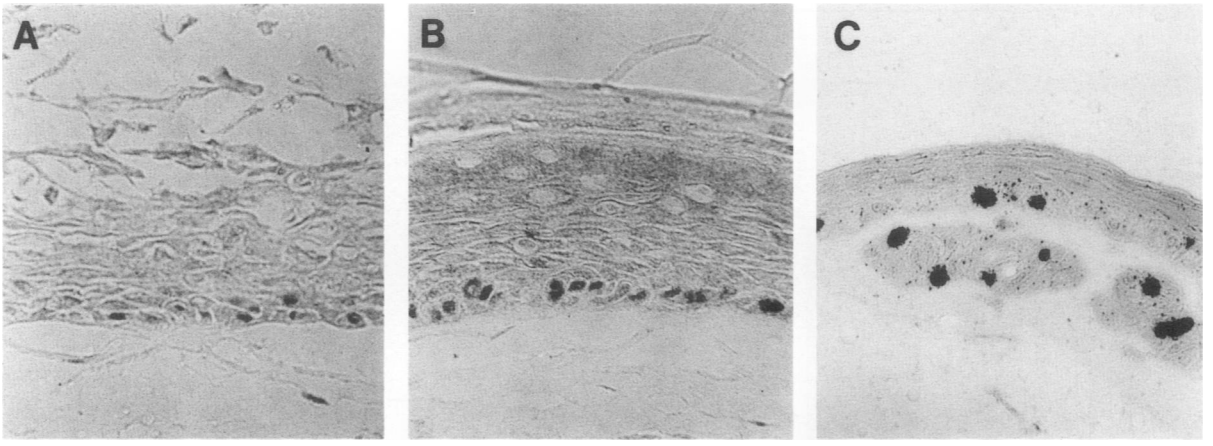

Figure 2. **Effects of TGFα on basal cell proliferation in human epidermal raft cultures.** Keratinocyte rafts were cultured for 3 wk at the air-liquid interface. At this time, cultures were either maintained on control medium (A) or exposed to TGFα or EGF for 12 h (B), 24 h (not shown), 48 h (not shown), or 72 h (C). Two hours before harvesting, [^{3}H]thymidine was added to the medium. After labeling, rafts were washed, fixed in Carnoy's solution, and embedded in paraffin. Sections (5 μm) were either stained with a monoclonal antibody against proliferating cell nuclear antigen (PCNA) (A and B) or exposed to Kodak NTB2 emulsion for 3 wk and then developed and stained with hematoxylin and eosin (C). Labeled cells were visualized by light microscopy. Note that PCNA-stained and [^{3}H]thymidine-labeled keratinocytes were restricted primarily to the innermost, basal layer (all conditions) or to the basal-like cells within the invading keratinocyte islands (>48 h post-TGFα treatment). (Magnification: ×155).

where swirling islands of keratinocytes appeared within the collagen lattice. In contrast, morphology in the suprabasal layers of the epidermal tissues seemed less affected, with the exception of an overall increase in cell layers and squames. The morphological changes induced by TGFα could also be generated by treating cells with comparable levels of EGF (not shown), consistent with the notion that these changes at least in part involved the EGF receptors on the surface of the basal epidermal cells.

As the length of exposure to TGFα increased, the intercellular contacts between basal-like cells became looser, and significant intercellular spaces became apparent (see Figure 1; 72-h TGFα treatment). This TGFα-mediated loosening of intercellular contacts was similar to that seen in transgenic mice overexpressing TGFα in the epidermis (Vassar and Fuchs, 1991). The loosening of intercellular connections was suggestive that the TGFα-treated basal cells were in a mobile state.

Because TGFα and EGF are known to enhance proliferation in keratinocytes cultured on plastic (Barrandon and Green, 1987), it seemed likely that the growth factor-induced increase in population of basal-like cells within the epidermal raft was due to hyperproliferation within the basal cell compartment. To test this possibility, we stained sections of untreated and TGFα-treated raft cultures with antibodies against proliferating cell nuclear antigen (PCNA), a DNA polymerase δ cofactor that is synthesized concomitantly with DNA (for review see Fairman, 1990; Baserga, 1991). We also labeled cultures with [^{3}H]thymidine and conducted autoradiography to detect the DNA synthesizing cells (see Materials and methods). As illustrated in Figure 2, PCNA-expressing and DNA-synthesizing cells were largely restricted to cells with basal-like morphology. This included those basal-like cells at the periphery of the keratinocyte islands appearing several days post-TGFα treatment (Figure 2C).

A compilation of the percentage of basal-like cells expressing PCNA in untreated and TGFα-treated cultures is given in Figure 3. As judged by these data, a threefold increase in proliferating cells occurred within 12 h post-TGFα-treatment. Thereafter, the proliferation rate returned to pretreatment levels.

The periphery of TGFα-induced epidermal islands express basal cell proteins, whereas the centers produce terminal differentiation-specific proteins

To investigate the biochemical nature of the TGFα-induced epidermal islands, we conducted an immunohistochemical analysis of tissue sections obtained from raft cultures treated with 30 ng/ml of TGFα for 0–3 d (Figure 4). For these studies, we chose antibodies against (1) involu-

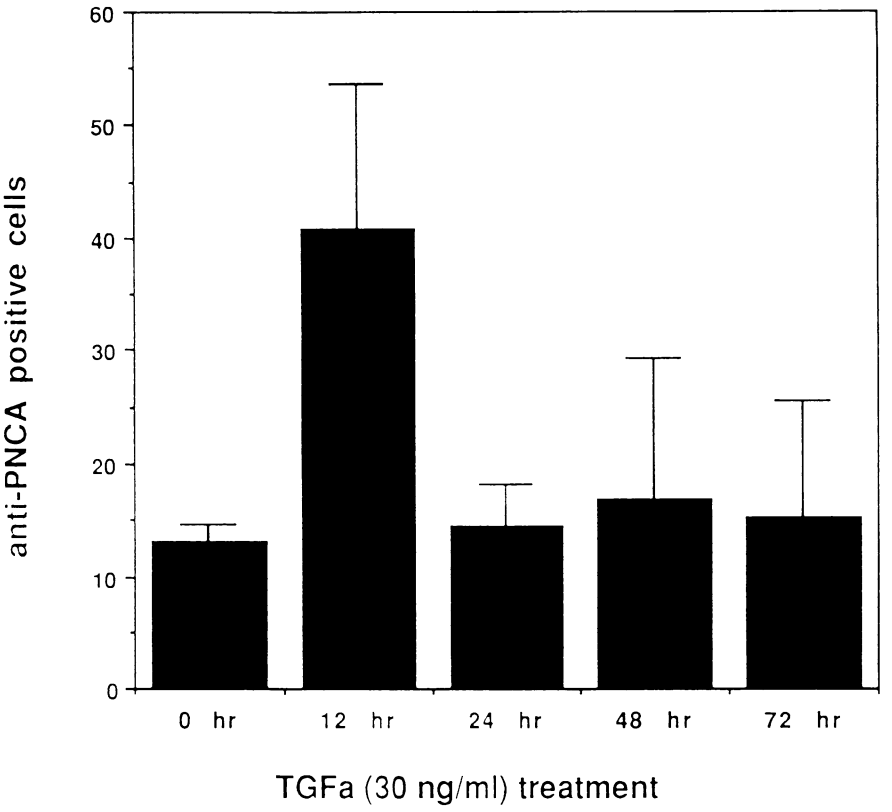

***Figure 3.* Quantitation of TGFα-induced basal cell proliferation.** Anti-PCNA-stained sections described in the legend to Figure 2 were quantitated to assess the percentage of basal-like cells expressing PCNA, i.e., proliferating, at 0, 12, 24, 48, and 72 h post-TGFα treatment. For each condition, the number of PCNA-expressing and nonexpressing basal cells were counted. As shown, a 3.1- +/− 0.3-fold stimulation in anti-PCNA-staining cells was observed in cultures treated for 12 h with TGFα.

crin, a cornified envelope protein expressed only in terminally differentiating keratinocytes (Rice and Green, 1979; Watt, 1984); (2) K6, a keratin that with its partner K16 is expressed in suprabasal epidermal cells undergoing hyperproliferation and/or exposed to a variety of growth factors (Weiss *et al.,* 1984; Mansbridge and Knapp, 1987; Mansbridge and Hanawalt, 1988; Stoler *et al.,* 1988; Kopan and Fuchs, 1989; Choi and Fuchs, 1990); and (3) fibronectin receptor, a biochemical marker for basal epidermal cells (Adams and Watt, 1990; Larjava *et al.,* 1990).

As expected, control cultures stained with anti-involucrin exhibited staining throughout the spinous and granular layers, with no staining of the basal layer (Figure 4; first frame of anti-INV series). TGFα-treated cultures also exhibited this staining, but in addition, showed staining in the center of the epidermal islands (see last two frames of anti-INV series, 48 and 72 h post TGFα-treatment). Neither the basal cells nor the peripheral cells of the islands stained with this antibody. The pattern of staining obtained with anti-K6 antiserum was similar to that obtained with anti-involucrin (see Figure 4). Collectively, these data suggested that once basal cells invaded and colonized the matrix, they were able to undergo at least some biochemical changes characteristic of terminal differentiation.

The patterns of staining observed with anti-involucrin and anti-K6 were directly opposite to those obtained with the anti-human fibronectin receptor (FN-R). Thus anti-FN-R staining was restricted largely to the basal layer of control cultures, with little or no staining in terminally differentiating cells (Figure 4; first frame of anti-FN-R series). At early times after TGFα treatment, the clusters of basal-like cells developing near the epidermal/dermal junction stained uniformly with anti-FN-R. At later times, after islands of epidermal cells had entered the collagen matrix, staining was largely restricted to the peripheral cells of the islands in addition to the basal layer of the epidermis (see Figure 4, anti-FN-R series 24–72 h post-TGFα treatment). Collectively, these data revealed that the periphery of the keratinocyte islands were basal-like in character, whereas the centers of the islands had undergone commitment to terminally differentiate. The invading islands were markedly similar in morphology and biochemistry to squamous cell carcinomas of the skin (see Fizpatrick *et al.,* 1987; Stoler *et al.,* 1988).

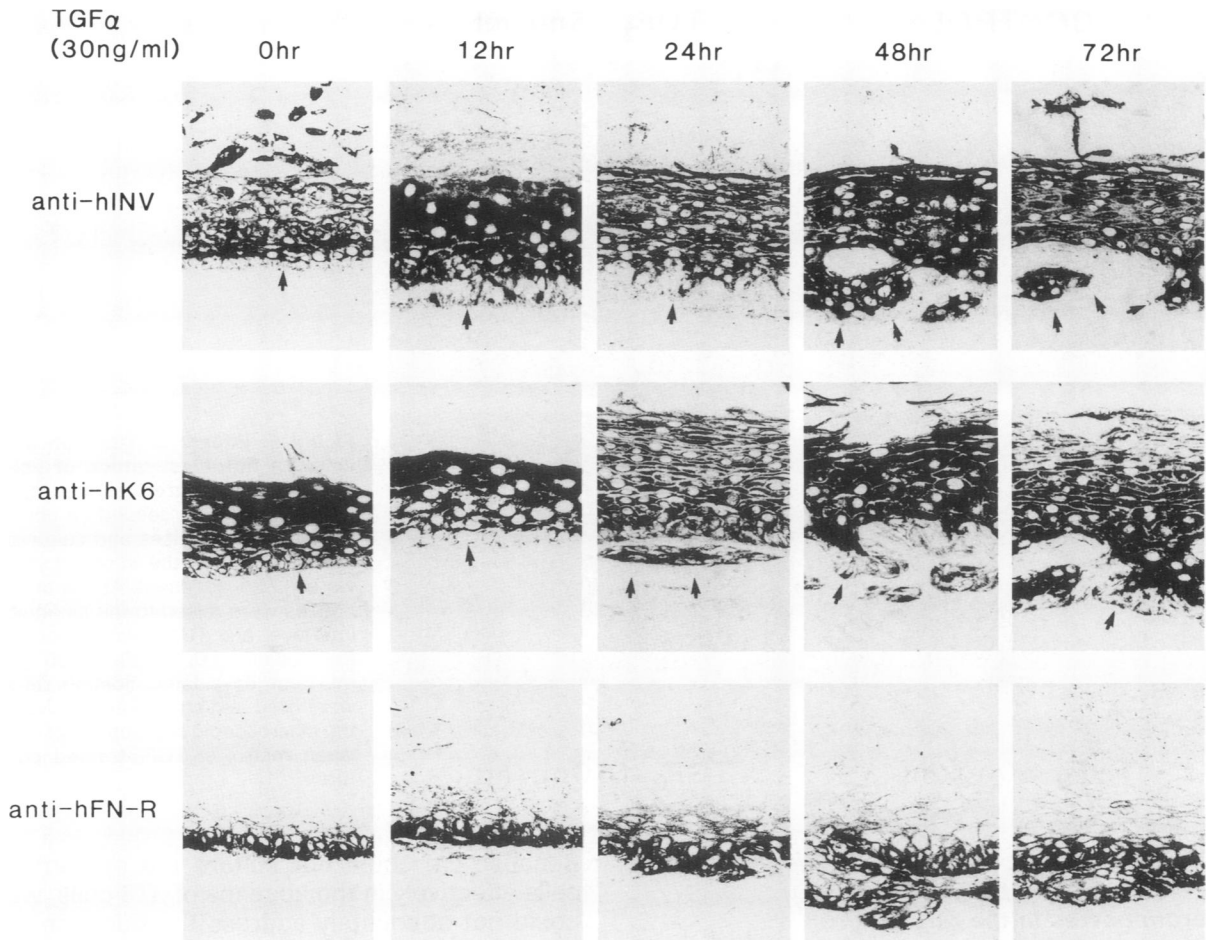

Figure 4. **Effects of TGFα on expression of differentiation-specific and basal cell markers in epidermal raft cultures.** Epidermal rafts were cultured for 3 wk at the air liquid interface. Rafts were then treated with TGFα (30 ng/ml) for 0, 12, 24, 48, and 72 h, followed by fixation in Carnoy's solution, embedding in paraffin, and sectioning (5 μm). For immunohistochemical labeling, sections were deparaffinized, rehydrated, and then exposed to antibodies against either differentiation-specific proteins (involucrin and keratin K6) or basal cell markers (fibronectin receptor). Antibodies were visualized by immunogold silver enhancement as described by Choi and Fuchs (1990). Antibodies used for immunostaining are given at the left of each set of sections (anti human involucrin, anti-hINV; anti human keratin 6, anti-hK6; anti human fibronectin receptor, anti-hFN-R). Arrows indicate cells that have invaded into the artificial dermis in the presence of TGFα. (Magnification: ×155).

TGFα causes an epidermal cell-mediated degradation of the collagen matrix

The invasion of TGFα-treated epidermal keratinocytes into the collagen matrix suggested that the matrix was locally degraded. This phenomenon was most striking when TGFα was added to the medium at early times, i.e., when epidermal raft cultures were first raised to the air-liquid interface. At this time, epidermal cultures were still subconfluent, with more mitotically active and less-differentiated cells than after 3 wk culturing at the air-liquid interface (Kopan *et al.,* 1987). Under these circumstances, considerably more lateral movement of cells was possible than in the heavily stratified and differentiating raft cultures. Remarkably, under these conditions, it was impossible to establish a raft culture because of massive degradation of the collagen-fibroblast lattice (Figure 5). Within a range of TGFα from 15–100 ng/ml, the higher the TGFα level, the more extensive the destruction of the collagen matrix. Similar results were also seen with EGF (not shown). Because matrix degradation was even more extensive in laterally migrating, subconfluent cultures than in well-stratified and differentiating raft cultures, it seemed possible that a common biochemical mechanism might be responsible for TGFα-mediated enhancement of both basal cell migration and cell invasion.

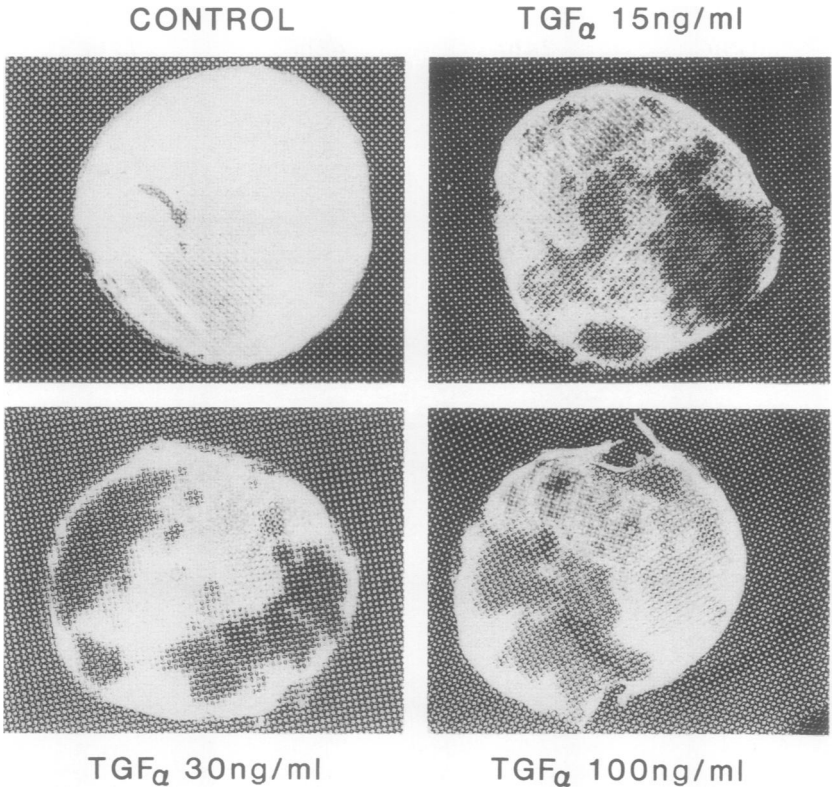

***Figure 5.* Effects of TGFα on the collagen fibroblast lattice of epidermal raft cultures.** Human epidermal cells were seeded on collagen-fibroblast lattices and cultured submerged until the keratinocytes were ~75% confluent. At this time, lattices were raised to the air-liquid interface, and TGFα was added to the medium at 0, 15, 30, or 100 ng/ml. Four days later, floating rafts were fixed and photographed. Note the macroscopic degradation of collagen matrix in TGFα-treated cultures.

TGFα-mediated collagen matrix degradation is dependent on the presence of keratinocytes in the raft culture

A priori, TGFα-mediated destruction of the collagen lattice might involve either the fibroblasts alone, the epidermal keratinocytes alone, or a combination of the two cell types. To distinguish between these possibilities, we repeated these experiments with two variations, namely 1) omitting the keratinocytes on the collagen lattice and 2) omitting the fibroblasts in the collagen-fibroblast lattice. When keratinocytes were omitted from the lattice, no destruction of the lattice occurred. Because 3T3 cells remained metabolically active in the absence of keratinocytes, this demonstrated convincingly that the degradation of the matrix was not solely due to the fibroblast component in the raft system and was dependent on the presence of human keratinocytes.

It was more difficult to assess unequivocally whether the keratinocytes degraded the matrix independently of the presence of fibroblasts. In the absence of fibroblasts, keratinocytes did not grow and differentiate well, a finding consistent with the hallmark studies of Rheinwald and Green (1975) and later studied extensively by others (Bohnert *et al.*, 1986; Finch *et al.*, 1989). Because we could not culture the epidermal cells effectively in the absence of 3T3 cells, we could not adequately address the question of whether keratinocytes alone can degrade the collagen lattice, or alternatively, whether there might be some as yet unidentified TGFα-inducible fibroblast factor involved in eliciting the keratinocyte response. Because EGF receptors exist on the surface of both human keratinocytes and 3T3 fibroblasts, a synergistic response is still a formal possibility.

TGFα-enhanced production of type I collagenase and gelatinase by epidermal raft cultures

To test whether the TGFα-mediated degradation of the collagen matrix might involve production of specific collagen-degrading enzymes, we analyzed proteins secreted by TGFα-treated, [^{35}S]methionine-labeled raft cultures. Culture medium of raft cultures treated for 3 d with 30 ng/ml TGFα was combined with a polyclonal antiserum specific for human type I collagenase (see Materials and methods for details). When subjected to immunoprecipitation, a complex formed that was then analyzed by SDS-PAGE (Figure 6). The complex resolved into two radiolabeled bands of 57 and 52 kDa (Figure 6A,

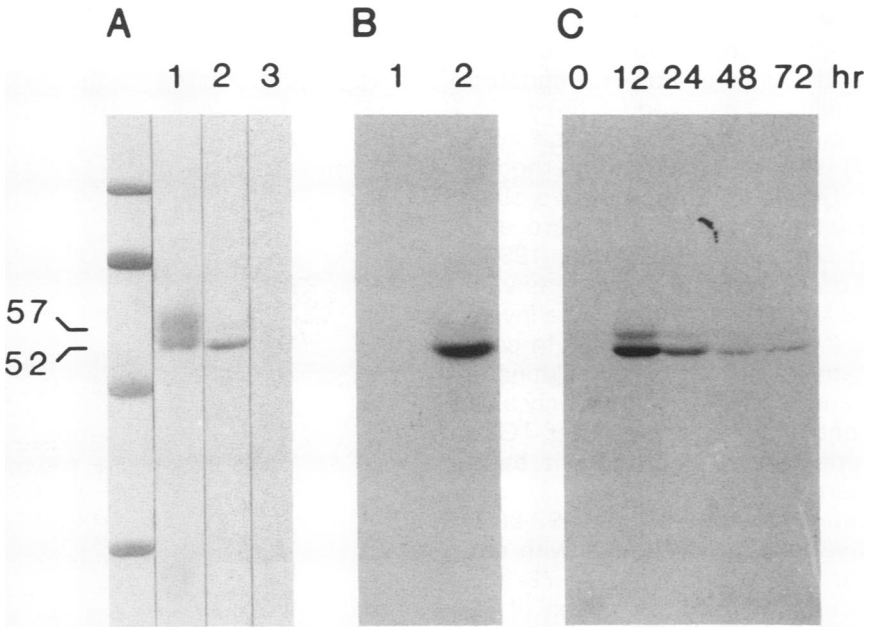

Figure 6. **Induction of type I collagenase by TGFα-treated raft cultures.** Human epidermal raft cultures were grown for 3 wk in the presence or absence of TGFα (30 ng/ml), followed by labeling with [^{35}S]methionine. Conditioned medium was then processed for immunoprecipitation with anti-type I collagenase antibody. The precipitate was analyzed by SDS-PAGE, followed by either staining (A, lanes 0 and 1) or fluorography and autoradiography (rest). (A) Lane 1, purified human type I collagenase (from fibroblasts); lane 2, immunoprecipitate of conditioned medium from TGFα-treated culture; lane 3, immunoprecipitate of conditioned medium from untreated culture. Molecular masses: type I collagenase (52 kDa), procollagenase (57 kDa) and protein standards (97, 66, 45 and 31 kDa). (B) Specificity of immunoprecipitation. Immunoprecipitation was repeated as outlined above, with the exception that unlabeled purified human type I collagenase (1 μg) was added to the conditioned medium before incubation with antibody. Lane 1, immunoprecipitate from conditioned medium of TGFα-treated cultures, after preincubation with unlabeled collagenase; lane 2, immunoprecipitate of conditioned medium of TGFα-treated cultures, without preincubation. (C) Time course of TGFα-mediated induction of type I collagenase by raft cultures. After 3 wk at the air liquid interface, human epidermal raft cultures were treated with TGFα (30 ng/ml) for 0, 12, 24, 48, and 72 h. Immunoprecipitations of conditioned media and SDS-PAGE analyses of the radiolabeled precipitates were performed as in A.

lane 2). These proteins migrated with the same electrophoretic mobility as purified human type I collagenase (Figure 6A, lane 1). Both bands were also detected in cultures treated with 30 ng/ml EGF (data not shown), but neither band was detected in cultures that were not exposed to TGFα (Figure 6A, lane 3). When purified (unlabeled) human type I collagenase was added to the extracts before immunoprecipitation, no radioactive bands were precipitated, indicating that purified collagenase competed efficiently with the radiolabeled bands for anti-collagenase antibody (Figure 6B, compare lanes 1, cold-competitor, vs. lane 2, no added competitor). These data indicated that the 52 and 57-kDa proteins detected in the medium of TGFα- or EGF-treated raft cultures represented human type I collagenase. Prior studies have demonstrated that the 52-kDa form corresponds to the active form of the enzyme and the 57-kDa form corresponds to the zymogen (Goldberg *et al.,* 1986; Petersen *et al.,* 1987). The zymogen is a latent precursor of active collagenase that can also be found in serum-containing organ cultures of skin as previously reported (Stricklin *et al.,* 1976).

If type I collagenase plays a role in TGFα-mediated enhanced migration/invasion of basal keratinocytes, then expression of collagenase might be expected to precede this event. To investigate this possibility, we examined the kinetics of type I collagenase induction after TGFα treatment (Figure 6C). In contrast to matrix invasion, which was optimal at ~24–72 h post-TGFα-treatment, collagenase induction coincided with enhanced epidermal proliferation and peaked within 12 h of TGFα addition. Collectively, these results are consistent with the notion that collagenase induction is a prerequisite to epidermal invasion into the collagen lattice.

Because systematic breakdown of type I collagen can also involve type IV collagenase (gelatinase), we wondered whether this enzyme might also be involved in the TGFα-induced

degradation of the lattice. To test this possibility, we resolved raft culture medium proteins by electrophoresis through gelatin-impregnated polyacrylamide gels and assayed for the ability of these proteins to degrade the gelatin substrate (Figure 7) (see Materials and methods). Interestingly, a 92-kDa band, one of two classical human gelatinase bands (Emonard and Grimaud, 1990; Matrisian and Hogan, 1990), was present in untreated raft cultures as well as TGFα-treated cultures at comparable levels (Figure 7, lanes 2 and 3, respectively). In contrast, however, the other classical gelatinase band, i.e., the 72-kDa band, was seen only as a faint band in control cultures, but after TGFα treatment it appeared as a prominent band (Figure 7, compare lanes 2 and 3).

The identification of the 72- and 92-kDa, bands as gelatinase is in agreement with our finding that the enzymatic activity of the 72- and 92-kDa bands was inhibited by addition of 50 mM EDTA to the gel (Figure 7, lanes 4–6). It is well known that human gelatinases are metalloproteinases and are consequently inhibited by EDTA (Emonard and Grimaud, 1990). Finally, to demonstrate that the enzymatic activities we detected were not merely general proteases, we substituted α-casein for gelatin and repeated the assay. In the presence of α-casein, no enzymatic activity was detected, thereby demonstrating that the enzymatic activities were specific for gelatin (not shown). Collectively, our data indicate that the raft cultures express the 92- and 72-kDa gelatinases and that the activity of the 72-kDa gelatinase is greatly elevated in response to TGFα treatment.

Because 3T3 fibroblasts could not elicit collagen matrix degradation in the absence of keratinocytes, it seemed likely that the keratinocytes might be responsible for the expression of type I collagenase and gelatinase. To test this possibility, we stained sections of untreated and TGFα-treated cultures with antibodies against type I collagenase and type IV collagenase (gelatinase) (Figure 8, top and bottom panels, respectively). Anti-human type I collagenase staining first appeared in the 12-h TGFα-treated cultures, and this persisted in cultures treated for 24, 48, and 72 h with the growth factor (Figure 8, top panels). Staining was prominent throughout the epidermal layers, indicating that irrespective of differentiation state, all TGFα-treated epidermal cells contain type I collagenase.

The pattern of anti-gelatinase staining (Figure 8, bottom panels) was distinctly different from that of anti-type I collagenase (Figure 8, top panels). Thus staining with an anti-human type IV collagenase antiserum revealed staining predominantly at the interface of the matrix and basal layer. Staining of the leading edges of the invading islands of keratinocytes was always less than that in the basal layer (see 48 and 72 h TGFα-treated cultures), suggesting that these

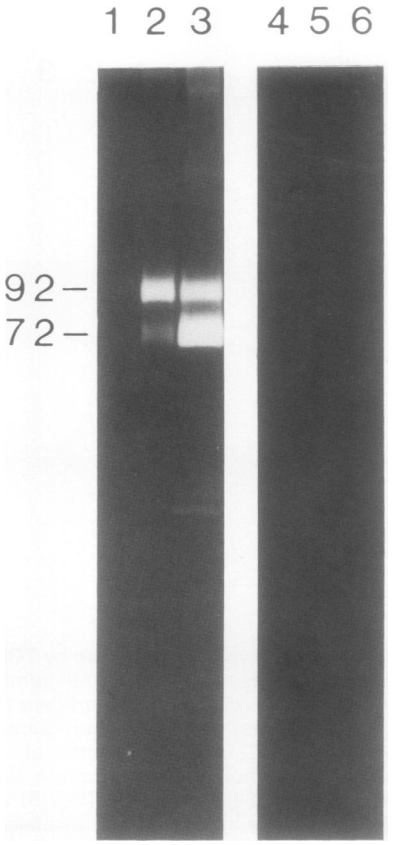

Figure 7. **Effects of TGFα on gelatinase activity in human epidermal raft cultures and in collagen/fibroblast lattices.** Human epidermal raft cultures or collagen/fibroblast lattices (i.e., raft cultures without epidermal cells) were grown for 3 wk at the air-liquid interface. Cultures were then treated with 0 or 30 ng/ml TGFα for 48 h. Conditioned medium was taken from the cultures as described in the legend to Figure 6. (A) Analysis of gelatinase activity. Aliquots (10 μl) of condition medium, from TGFα-treated collagen/fibroblast lattices (lane 1), untreated epidermal raft cultures (lane 2), or TGFα-treated epidermal raft cultures (lane 3) were subjected to electrophoresis through polyacrylamide gels impregnated with gelatin as described in Materials and methods. After electrophoresis, gels were washed, incubated in calcium assay buffer, and stained (see Materials and methods). Zones of gelatin lysis appear against a black background of Coomassie Blue-stained gelatin. (B) Effects of EDTA on gelatinase activity. Aliquots of conditioned medium from collagen/fibroblast lattices (lane 4), untreated epidermal raft cultures (lane 5), and TGFα-treated epidermal raft cultures (lane 6) were subjected to electrophoresis and processed as above, with the exception that 50 mM EDTA was added to the calcium assay buffer.

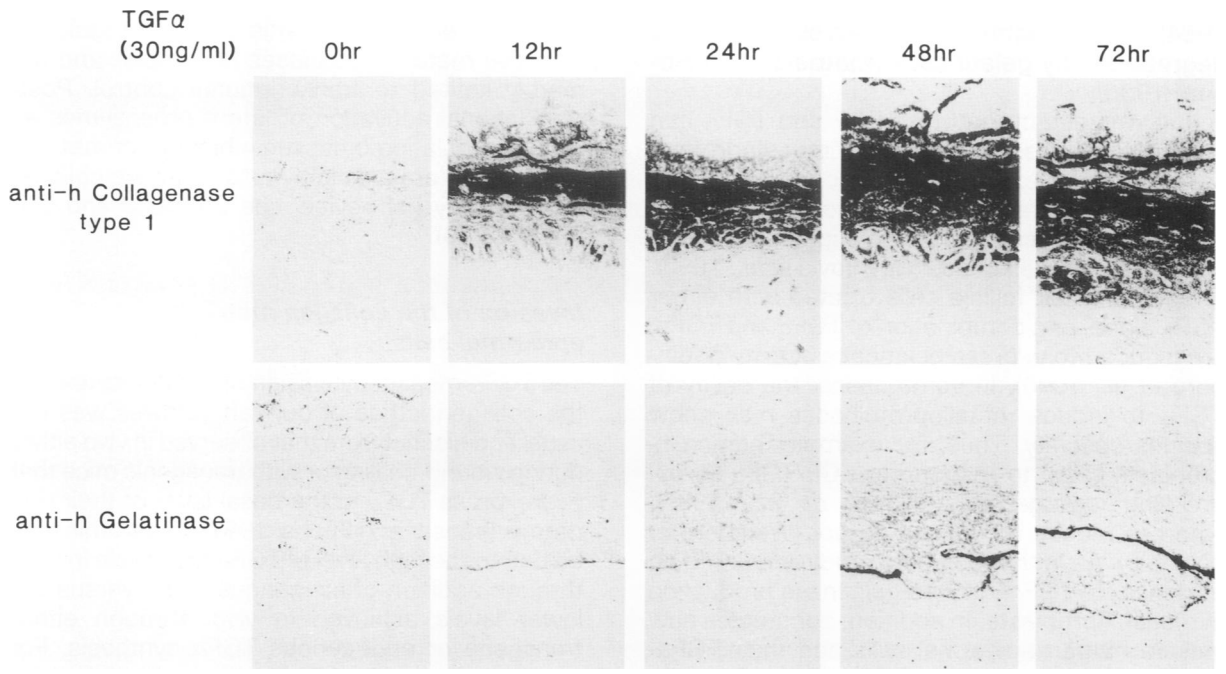

Figure 8. **Distribution of type I collagenase and type IV collagenase (gelatinase) in TGFα-treated epidermal raft cultures.** Epidermal rafts were cultured for 3 wk at the air liquid interface. After this time, rafts were treated with TGFα (30 ng/ml) for 0, 12, 24, 48, and 72 h. Rafts were then fixed in Carnoy's solution, embedded in paraffin, and sectioned (5 μm). For immunohistochemical labeling, sections were deparaffinized, rehydrated, and then exposed to antisera against either rabbit anti-human type I collagenase (anti-h collagenase) or rabbit anti-human type IV collagenase (anti-h gelatinase). Antibody staining was visualized by the immunogold silver enhancement technique as described by Choi and Fuchs (1990). Antibodies for immunostaining are indicated at the left of each set of sections. (Magnification: ×155).

cells may produce less gelatinase than basal keratinocytes.

Discussion

Cell migration plays an essential role in normal skin physiology, particularly during wound-healing and re-epithelialization. Although it has long been suspected that basal keratinocytes and growth factors are likely to be involved in this process, the underlying molecular mechanism remains complex and poorly understood.

In a pioneering study by Barrandon and Green (1987), it was shown that human keratinocytes cultured on plastic respond to TGFα by increasing their proliferation as a consequence of enhancing their ability to migrate. In our studies, we were unable to analyze in detail the effects of TGFα on lateral migration of epidermal keratinocytes, because of the high level of collagen-matrix degrading activity present in subconfluent raft cultures treated with this growth factor. However, this finding did suggest that migrating epidermal cells may be capable of producing very high levels of collagen degrading activities, a feature that may contribute to the TGFα-mediated enhancement of epidermal migration observed by Barrandon and Green.

The features of the raft culture system also enabled us to investigate the role of TGFα on the physiology of a three-dimensional skin system. We have demonstrated that the morphological changes elicited by TGFα are largely confined to the basal cells within the population. In addition, we have shown that when TGFα levels are elevated, even relatively constrained basal cells in the centers of the raft cultures are activated to enhance their proliferation and degrade and invade the underlying collagen matrix. Degradation of the matrix is preceded by production of elevated levels of type I collagenase and gelatinase. Thus TGFα and collagen-degrading enzymes appear to be involved in matrix degradation, cell invasion, and epidermal tissue remodeling.

Collagenases and cell invasion

Collagens are resistant to degradation by most proteinases. Interstitial collagenase or matrix metalloproteinase-1 initiates degradation by cleaving the triple helical molecule at a single site, generating fragments that spontaneously denature at body temperature (Harris *et al.*,

1984). These fragments are then susceptible to degradation by gelatinases (Matrisian and Hogan, 1990).

It is well established that EGF and TGFα can influence expression of matrix metalloproteinases. Thus, for example, transfection of a TGFα transgene into rat bladder carcinoma cells (NBT-11) leads to secretion of the higher molecular weight form of gelatinase (Gavrilovic *et al.*, 1990). In addition, hair follicle cells treated with either TGFα alone, or a combination of TGFα and TGFβ, are induced to express gelatinase activity (Weinberg *et al.*, 1990). In some cases, the ability of TGFα to induce metalloproteinases may show species specifity. Thus, for example, human fibroblasts seem to respond to EGF/TGFα by expressing collagenases (Chua *et al.*, 1985), whereas mouse fibroblasts do not (Weinberg *et al.*, 1990). As in the Weinberg study, we found no evidence for TGFα-induced gelatinase production in mouse fibroblasts. In addition, our studies now include human epidermal cells and their TGFα-mediated induction of type I and type IV collagenases on a growing list of cell types responding to TGFα by inducing metalloproteinases.

It seems certain that interaction of EGF/TGFα with its surface receptor constitutes the initial event in the pathway leading to metalloproteinase production by raft cultures. Although the kinetics of the response favor a relatively straightforward mechanism, it is interesting to note that both fibroblasts and keratinocytes have EGF receptors, and at least in other cell types, collagenase can be regulated by a number of other growth factors, including TNFα (Dayer *et al.*, 1985) and interleukin 1 (Postlewaite *et al.*, 1983; for review see Kupper, 1990), both of which are known to be inducible in keratinocytes (for review see McKenzie and Sauder, 1990). Some examples of epithelial-mesenchymal influences on collagenase production have been reported previously for other systems (Johnson-Muller and Gross, 1978; Johnson-Wint and Bauer, 1985, 1988). Thus we cannot exclude the possibility that fibroblasts might play an indirect role in the TGFα-mediated induction of metalloproteinases in epidermal raft cultures.

That a number of different cell types can induce collagenase in response to various growth factors is perhaps not surprising, given the mechanisms underlying control of the human collagenase gene. Recent studies have shown that this gene contains a regulatory site for the family of AP-1 transcription factors (Angel *et al.*, 1987; Gutman and Wasylyk, 1990). Because one of the AP-1 transcription factors is the growth factor-inducible protein c-*fos*, this gene may be generally controlled by factors that influence cell growth (see Schonthal *et al.*, 1988). This said, the regulation of active metalloproteinases is complex and not merely limited to transcriptional control. Post-translational activation of latent proenzymes as well as regulation by tissue inhibitors of metalloproteinases are also known to influence collagenase activity (for review see Emonard and Grimaud, 1990).

Invasion of the collagen matrix by epidermal cells

The TGFα-mediated invasion of keratinocytes into the collagen lattice of our raft cultures was unusual and distinct from that observed in vivo either during wound-healing or with transgenic mice that overexpress TGFα in the basal layer of their epidermis (Vassar and Fuchs, 1991). In part this may be due to the high level of TGFα attainable in vitro through addition of exogenous TGFα versus the lower levels achieved in vivo, through either transgene or endogenous TGFα synthesis. For example, the levels of TGFα attained with various founder mice harboring a rat TGFα transgene driven by a basal keratin promoter were only 2–8 times higher than physiological levels (Vassar and Fuchs, 1991), whereas in vitro, we have used 15–30 times these levels. An alternative explanation is that raft cultures do not produce as organized a basement membrane as basal cells in vivo and that the basal lamina in vivo provides a greater barrier to TGFα-induced basal cell migration than that which exists in the raft cultures. Thus, despite the ability of raft cultures to synthesize and deposit laminin, fibronectin, and collagen type IV (Woodley *et al.*, 1980; Liscia *et al.*, 1988; Choi and Fuchs, 1990), differences in this lamina may nevertheless be responsible for permitting dermal invasion in culture, a phenomenon that does not take place during wound-healing or in transgenic mice, when localized concentrations of TGFα in the skin are elevated.

Irrespective of the relation between the in vivo and in vitro effects of TGFα-induced cell migration, the invasive behavior of TGFα-treated, collagenase-expressing keratinocytes was intriguing in light of recent studies correlating increased expression of extracellular matrix-degrading metalloproteinases and acquisition of a malignant phenotype (for review, see Liotta and Stetler-Stevenson, 1989). This said, the effects elicited in our cultures were transient and thus in contrast to tumor invasion and growth; epidermal proliferation and collagenase production peaked within 12 h after TGFα addition to the culture medium, and the resulting invasion of basal keratinocytes into the collagen lattice was optimal at ~48–72

h post-TGFα-treatment. Collectively, our findings are consistent with the notion that TGFα by itself does not lead to neoplastic progression of epidermal cells (Finzi *et al.,* 1988).

Although TGFα may not be sufficient for transformation and tumorigenesis in epidermal cells, the morphological and biochemical resemblance between papillomas or squamous cell carcinomas and our TGFα-treated raft cultures was striking (see Fitzpatrick *et al.,* 1987). This was especially intriguing in light of recent transgenic mouse studies demonstrating that overexpression of TGFα in adult mouse epidermis led to papilloma-like formations but only on wounding (Vassar and Fuchs, 1990). Our present studies provide further support to the notion that TGFα at prolonged elevated levels may elicit certain responses that may act in concert with other factors/responses to contribute to a transformed state.

Conclusions

In summary, we have provided convincing evidence that TGFα elicits a transient increase in cell proliferation and collagenase and gelatinase production in human epidermal raft cultures. This response is followed by degradation of the collagen lattice concomitant with an increase in basal cell migration into the lattice. The extent to which additional factors might be involved in the process of epidermal cell migration/invasion and tissue remodeling (see, e.g., Liotta *et al.,* 1986; Gherardi *et al.,* 1989; Albelda and Buck, 1990; Guo *et al.,* 1990) awaits further investigation.

Materials and methods

Preparation of raft cultures

Collagen/fibroblast lattices were prepared using Cell Matrix A (Nitta Gelatin, Tokyo, Japan) as a source of type I collagen, as described previously (Asselineau *et al.,* 1986). Lattices were seeded with 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). Cells were grown submerged in medium for 7 d and then floated for 2–3 wk on stainless steel grids. Cultures were maintained with growth medium consisting of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 at a 3:1 ratio. Medium was supplemented with 15% fetal bovine serum (Hyclone, Logan, UT) supplemented with 1×10^{-10} M cholera toxin, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 2×10^{-11} M triiodothyronine. TGFα (Lot #90-0686; Collaborative Research, Bedford, MA) or recombinant EGF (Amgen Corp., Thousand Oaks, CA) was added at 0–100 ng/ml as indicated in the text, and medium was changed every 2 d. TGFα was stored at −70°C as 100× stocks in 0.1 mg/ml bovine serum albumin. Control cultures received a 1:100 dilution of 0.1 mg/ml bovine serum albumin.

Measurements of cell proliferation

[^{3}H]thymidine labeling, fixation, autoradiography, and hematoxylin/eosin staining of raft cultures. 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 phosphate-buffered saline, cultures were fixed in Carnoy's (6:3:1 of ethanol, chloroform, and 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.

PCNA staining/analysis. Proliferating cells were identified immunohistochemically using monoclonal antibodies against PCNA (see below). For each condition of TGFα treatment, positive and negative basal cells in 10 fields were counted. Results were tabulated and expressed as the means ± SD.

Immunohistochemistry

Fixed, paraffin-embedded sections (5 μm) were hydrated before immunohistochemical staining. Staining was carried out as described by Kopan and Fuchs (1989). Antisera and dilutions used were mouse monoclonal antibodies against PCNA (1:40 dilution) (Boehringer Mannheim Biochemicals, Indianapolis, IN), rabbit polyclonal anti-human keratin 6 antisera anti-hK6 (1:100) (Stoler *et al.,* 1988), rabbit polyclonal anti-human anti-FN-R (1:200) (Chemicon Int. Inc., Temecula, CA), rabbit polyclonal antisera against human involucrin (1:20) (Biomedical Technologies, Inc., Stoughton, MA), rabbit polyclonal antiserum against human type I collagenase (1:200) (Peterson *et al.,* 1989), and rabbit polyclonal antiserum against human type IV collagenase (72 kDa) (1:200) (Monteagudo *et al.,* 1990). After incubation with primary antisera, slides were subjected to immunogold enhancement as described by Kopan and Fuchs (1989).

Immunoprecipitation

Untreated and TGFα-treated raft cultures were labeled overnight with 10 μCi/ml of [^{35}S]methionine (1163 Ci/mmol, New England Research Products, Boston, MA). Media was subjected to centrifugation at 10 000 × *g* for 20 min at 4°C to remove any debris. Supernatants (5-ml aliquots) were incubated with 1-μl polyclonal rabbit anti-human collagenase antibody (Petersen *et al.,* 1989) for 12–15 h at 4°C. Protein A sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was then added and after an additional 2-h incubation at 4°C, samples were subjected to centrifugation at 10 000 × *g* for 20 min. The pellet was then washed extensively with 10 mM tris(hydroxymethyl)aminomethane (Tris) HCl, 0.15 M NaCl, 3 mg/ml nonidet P-40, pH 7.4, followed by boiling in the presence of SDS-PAGE gel sample buffer. Samples were subjected to electrophoresis through 8.5% polyacrylamide gels. For fluorography, gels were treated with Amplify (Amersham) for 30 min, dried, and exposed to X-OMAT AR film (Eastman Kodak) at −70°C for 1–3 d.

Gelatin substrate gel electrophoresis

Electrophoresis was carried out according to the method of Umeroni and Werb (1986), with 10% SDS-PAGE containing either gelatin (test gel; Sigma Chemical Co., St. Louis, MO) or casein (control gel; Sigma) at 2 mg/ml. Aliquots (10 μl) of culture media were subjected to electrophoresis, and gels were then incubated in 2% Triton X-100 for 30 min (2 changes) at 37°C with mild shaking. Gels were rinsed thoroughly with glass-distilled water and then incubated in 50 mM Tris/HCl pH 7.4 containing 30 mM $CaCl_2$, and where indicated, 50 mM EDTA, for 15 h at 37°C on a shaker. After rinsing with distilled water several times, gels were stained with Coomassie Brilliant Blue.

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