Cytokine-Induced Expression of Transforming Growth Factor-α and the Epidermal Growth Factor Receptor in Neonatal Skin Explants

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Hyperproliferative diseases of the epidermal keratinocytes, such as psoriasis vulgaris, are characterized by overexpression and altered distribution of the epidermal growth factor/transforming growth factor (EGF/TGF)-α receptor, and of TGF-α itself. It is believed that overexpression of this ligand/receptor system contributes to the hyperproliferative state of keratinocytes in an autocrine fashion. However, little is known about the factors that regulate expression of the EGF/TGF-α receptor, as well as expression of TGF-α in stratified epithelium. We examined modulation of the immunoreactive EGF/TGF-α receptor and TGF-α expression in normal neonatal foreskin explants by a variety of cytokines present in psoriatic lesions. Human (hu) recombinant (r) tumor necrosis factor (TNF)-α and interferon (IFN)-γ induced EGF/TGF-α receptor and TGF-α expression by keratinocytes as determined by immunohistochemistry. Neutralizing antibodies to TNF-α and IFN-γ inhibited upregulation of EGF/TGF-α receptors and TGF-α by the respective cytokines. Interleukin (IL)-8 induced expression of TGF-α, but not of its receptor. Other cytokines (TNF-β, IFN-β, IL-1α, IL-2, IL-3, IL-5, IL-6, granulocyte/macrophage colony-stimulating factor, and macrophage colony-stimulating factor) did not alter the expression patterns of EGF/TGF-α receptors or TGF-α in normal neonatal skin explants. These experiments demonstrate that specific cytokines known to be present in psoriatic lesions can induce normal epidermis to express TGF-α and its receptor in a pattern similar to that observed in psoriatic skin. J Invest Dermatol 99:350–356, 1992

The epidermis is one of the few tissues that is mitotically active throughout the lifespan of the organism. Two structurally and functionally related growth factors, epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), appear to contribute to the proliferation of normal human keratinocytes during embryologic development and in tissue culture [1,2]. Addition of TGF-α to cultured keratinocytes not only stimulates growth, but also induces endogenous TGF-α production, which is likely to amplify the growth response to exogenous TGF-α [3]. Proliferative effects of both growth factors, EGF and TGF-α, are mediated by activation of the tyrosine kinase moiety of the EGF/TGF-α receptor [4,5] that is expressed on the surface of keratinocytes in vivo and in vitro [6]. Inhibition of the EGF/TGF-α receptor tyrosine kinase activity results in inhibition of keratinocyte proliferation [4]. Conversely, constitutive overexpression of TGF-α in the skin of transgenic mice, under the control of the human keratin K14 promoter, leads to epidermal thickening, reflecting increases in keratinocyte numbers and cell hypertrophy [7]. These results indicate an autocrine role for TGF-α in keratinocyte growth in vivo.

Activation of the TGF-α–dependent autocrine loop in keratinocytes may be regulated by cytokines. Various cytokines, such as interleukin (IL)-1 [8], tumor necrosis factor-alpha (TNF-α) [9], and interferon-gamma (IFN-γ) [10,11], have been shown to increase steady-state TGF-α mRNA levels in cultured keratinocytes. In addition, IFN-γ appears to modulate EGF-receptor protein expression of cultured keratinocytes. Both increased [12] and decreased [13] EGF-receptor expression was observed after IFN-γ treatment of keratinocytes in culture.

However, studies with cultured keratinocytes cannot mimic the potential complex interactions between cells of different types nor between keratinocytes in distinct differentiation states. We have, therefore, examined the effect of various cytokines on the pattern of expression of TGF-α and the EGF/TGF-α receptor in organ cultures of normal neonatal skin.

MATERIALS AND METHODS

Organ Culture Short-term organ cultures of skin samples were carried out as described [14,15] with minor modifications. Briefly, neonatal foreskins, obtained from four donors, were immersed in MCDB 153 medium containing 0.03 mM calcium, amino acids, ethanolamine, phosphoethanolamine, and hydrocortisone as described, but no pituitary extract or polypeptide growth factors (base medium) [16]. After removal of fatty tissue from the dermal side, skin samples were cut into approximately 2-mm square pieces and placed epidermal surface up in wells of 6-well tissue culture plates.
Base medium (approximately 800 µl) containing cytokines was added. The epidermal surface of explants remained uncovered by culture medium. Explants were incubated at 37 °C in a humidified incubator. In control cultures medium was not supplemented with cytokines, or cytokines were mixed with neutralizing antibodies prior to addition to cultures. Explants were harvested after 24 h, embedded in OCT compound (Tissue-Tek, Miles, Inc., Elkhart, IN), and snap-frozen at -70 °C.

Cytokines, Antibodies, and Immunohistochemistry Explants were incubated with the following cytokines at different concentrations: human (hu) recombinant (r) IL-8 (Pepro Tech Inc., Rocky Hill, NJ) at 5, 10, and 25 ng/ml; hu rTNF-α (Genentech, San Francisco, CA) at 500, 1,000, and 10,000 U/ml; hu rIFN-γ (Genentech) at 500, 1,000, and 10,000 U/ml; hu rIL-1α (Genetics Institute, Cambridge, MA) at 25, 50, and 100 U/ml; hu r granulocyte/macrophage colony-stimulating factor (Genetics Institute) at 1,000, and 10,000 U/ml; hu r macrophage colony-stimulating factor (Genetics Institute) at 1,000, and 10,000 U/ml; hu rIL-3 (Genetics Institute) at 700 and 1,400 U/ml; hu rIL-5 (Genetics Institute) at 50, 100, and 500 U/ml; hu rIL-6 (Genetics Institute) at 10, 20, and 40 U/ml (Genetics Institute); hu rTNF-β (Cetus Corp., Emeryville, CA) at 1,000, and 10,000 U/ml; and hu rIFN-β (Genentech) at 1,000, and 10,000 U/ml. For blocking experiments, neutralizing monoclonal antibodies (MoAb) to TNF-α (MoAb 154.2.1) and IFN-γ (mAb B33.31), kindly provided by Dr. G. Trinchieri, were used simultaneously with cytokines at 1:200 and 1:400 dilutions of ascitic fluid.

For immunohistochemical staining, unfixed, 4-6 µm thick frozen sections were incubated for 1 h with MoAb recognizing hu TGF-α (Ab2; Oncogene Science, Inc., Manhasset, NY) and the EGF/TGF-α receptor (MoAb 425) [17] at 5 and 10 µg/ml, respectively. Ab2 recognizes hu TGF-α in its free and receptor-bound form (communication by Oncogene Science). MoAb 425 [18] binds specifically to a protein epitope on the external domain of the hu EGF/TGF-α receptor. It immunoprecipitates a 170-kDa protein from 35S-labeled A 431 cells, recognizes a 3P-labeled 170-kDa protein in EGF-stimulated A 431 squamous carcinoma cells and plasmatic membranes, and binds to the proteolytically cleaved 110-kDa external domain of hu EGF/TGF-α receptor [18]. It recognizes the occupied as well as the unoccupied EGF/TGF-α receptor because it immunoprecipitates a 125I-EGF/170-kDa complex from various human cell lines and tissues [18]. Scatchard analysis demonstrated that MoAb 425 binds to high- and low-affinity EGF/TGF-α receptors with kinetics very similar to EGF itself [18]. No protein other than the EGF/TGF-α receptor is detected by MoAb 425 in immunoprecipitation analyses of cell membrane preparations of A 431 squamous carcinoma cells.

Antibody binding was visualized using a fluorescein isothiocyanate–labeled goat anti-mouse immunoglobulin G at 1:100 dilution (Jackson ImmunoResearch, West Grove, PA). Cytokine–treated organ cultures were evaluated parallel to samples incubated with cytokines and antibodies.

In order to demonstrate viability of the organ cultures, immunoperoxidase staining of sections was performed using antibodies against endothelial cell adhesion molecules, one of which has been previously shown to be upregulated by cytokines in organ culture [14]. Control and TNFα-stimulated cultures were thus stained with an anti–PECAM-1/endoCAM MoAb that specifically identifies all endothelial cells in tissue sections [19,20] and with an anti–E-selectin (ELAM-1) antibody H4/18 [14], kindly provided by Dr. Michael Bevilacqua. The bound first antibody was detected by the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA) with AEC as chromagen. No counterstain was applied in order to increase sensitivity. Photographs were taken with a Zeiss Photomicroscope III using Kodak T-MAX 100 film.

RESULTS

Morphology and Viability of Skin Explants After 24 h of organ culture, the keratinocytes appeared intact and similar in appearance to normal skin after hematoxylin-eosin staining (Fig 1A).

To confirm cellular viability, sections were stained with antibodies that specifically recognize endothelial cell adhesion molecules. The entire vasculature of the graft was visualized using the anti–PECAM-1 MoAb (Fig 1B), and illustrates that the endothelium was intact and expressing normal cell surface antigens. To determine if these cells could respond to cytokine stimulation, sections of organ cultures exposed to base medium (Fig 1C) and cultures exposed to 10,000 units of TNF-α (Fig 1D) for 24 h were stained for the presence of E-selectin. Although E-selectin was completely absent in control sections (Fig 1C), a small percentage of vessels in the TNF-α–treated culture showed clear expression of E-selectin (arrowheads in Fig 1D). This relatively weak, but significant, upregulation of E-selectin at 24 h is similar to that reported by Messadi et al [14].

Effects of TNF-α and IFN-γ on TGF-α and EGF/TGF-α Receptor Expression in Skin Explants Following 24 h of incubation with MCDB 153 base medium containing 0.03 mM calcium, amino acids, ethanolamine, phosphoethanolamine, and hydrocortisone, but no cytokines, a low-grade (2+) expression of TGF-α was detected predominantly in the intermediate and upper layers and, to a lesser degree, in the basal-suprabasal region of skin explants (Figs 2A and 4A). After incubating samples with 1000 U/ml TNF-α for 24 h, a marked increase in TGF-α expression (4+) was noted in the intermediate and upper skin layers (Fig 2B). Also, in a few sectors, basal and suprabasal keratinocytes showed increased staining (2+) for TGF-α (Fig 2B). Upregulation of TGF-α was also observed when samples were treated with 500 U/ml of TNF-α (not shown). Expression of TGF-α was somewhat reduced, as compared to untreated controls (Fig 2C), when explants were incubated simultaneously with TNF-α and MoAb 154.2.1, a neutralizing antibody to TNF-α.

Incubation with IFN-γ at 1000 U/ml, but not at 500 U/ml, similarly resulted in higher expression of TGF-α, although staining intensity was less (3+) than that observed after addition of TNF-α (Fig 1D). Incubation with IFN-γ in the presence of MoAb B33.31, a neutralizing antibody to IFN-γ, prevented the upregulation of TGF-α expression (Fig 1E).

Expression of the EGF/TGF-α receptor was confined to basal-suprabasal cells in untreated control samples (Fig 3A). Following incubation with either TNF-α (500 or 1000 U/ml) or IFN-γ (1000 U/ml), keratinocytes in all layers of the epidermis were evenly stained for the EGF/TGF-α receptor (Fig 3B, D). Similar to results described for TGF-α, upregulation of the EGF/TGF-α receptor could not be observed when TNF-α- or IFN-γ-induced effects were inhibited with the respective neutralizing antibody (Fig 3C, E). Furthermore, treatment of cultures with the TNF-α antibody alone resulted in reduced staining for the EGF/TGF-α receptor when compared to untreated controls, suggesting that endogenous TNF-α may contribute to EGF/TGF-α receptor expression.

Effect of IL-8 on TGF-α and EGF/TGF-α Receptor Expression in Skin Explants Treatment of skin explants with 5 ng/ml hu rIL-8 resulted in a marked upregulation of TGF-α in all layers of the epidermis (4+ vs. 1 or 2+ in controls) (Fig 4A, B). Similarly, results obtained with IFN-γ, basal and suprabasal cells stained only weakly for TGF-α in control cultures. No modulation of EGF/TGF-α receptor expression by IL-8 was observed in three consecutive experiments carried out on tissues from different donors (not shown).

Effects of Other Cytokines on TGF-α and EGF/TGF-α Receptor Expression in Skin Explants No stimulation of TGF-α and EGF/TGF-α receptor expression was detected in four consecutive experiments using material from different donors when skin explants were treated with hu rIL-1α, IL-3, IL-5, or IL-6. Similarly, TNF-β, IFN-β, macrophage/colon-stimulating factor, and granulocyte macrophage/colon-stimulating factor were ineffective when tested in three separate experiments.
Figure 1. Morphology and viability of skin explants. Skin explants were stained after 24 h of organ culture. Hematoxylin-eosin staining reveals that the keratinocytes are normal in appearance (A). The microvasculature of the explants were visualized by immunohistochemical staining with the endothelial cell-specific antibody PECAM-1 (B). The viability of the cells within the explants was determined by exposing organ cultures to base medium or 10,000 units of TNF-α for 24 h and then staining for the inducible endothelial cell adhesion molecule, E-selectin. No expression of E-selectin was seen on the vessels of the control culture (C). In contrast, clear expression of E-selectin (arrowheads) was seen in some of the vessels in the TNF-α-treated graph (D). Bars, 50 μm.
Figure 2. TGF-α expression of neonatal skin explant cultures treated with IFN-γ or TNF-α. Explants were incubated in MCDB 153 base medium with no cytokine additive (A), or in the presence of TNF-α (B) or IFN-γ (D); on B, arrowheads point at TGF-α-positive areas in the basal-suprabasal region. C and E show inhibition of cytokine-induced TGF-α expression by neutralizing antibodies to TNF-α and IFN-γ, respectively. Bars, 30 μm.
Figure 3. EGF-receptor expression of neonatal skin explant cultures treated with IFN-γ or TNF-α. Explants were incubated in MCDB 153 base medium with no cytokine additive (A), or in the presence of TNF-α (B) or IFN-γ (D). C and E show inhibition of cytokine-induced EGF-receptor expression by neutralizing antibodies to TNF-α and IFN-γ, respectively. Bars, 30 μm.
Expression of immunoreactive TGF-α protein has been reported to be generally enhanced, but not significantly redistributed, in active psoriatic lesions when compared to normal skin or uninvolved skin from psoriatic patients [23–25]. This finding is reflected by elevated TGF-α mRNA levels in psoriatic plaques [10,24]. Enhanced expression of TGF-α mRNA [24] and protein [25] was observed, particularly in psoriatic suprabasal keratinocytes. This is the same location in which we found the strongest cytokine-induced increase of immunoreactive TGF-α in normal epidermis. At variance with findings of Turbitt et al [24], TGF-α immunostaining of basal normal keratinocytes in control explants was weak. However, treatment with IL-8 led to detection of elevated TGF-α protein levels not only in the suprabasal stratum but also in basal keratinocytes.

Our results suggest that the pattern of the EGF/TGF-α receptor and TGF-α expression as observed in psoriasis is not unique to this disease, but can be induced in normal neonatal skin and, thus, may be comparable to a physiologic response to cytokine stimulation of keratinocytes. Activated immune cells such as T-lymphocytes, monocytes/macrophages, and Langerhans cells are obvious sources for cytokines such as IFN-γ and TNF-α in active psoriatic lesions. In fact, Nickoloff et al [9] recently described that TNF-α is expressed predominantly by macrophages in the papillary dermis of psoriatic lesions, and focally by epidermal Langerhans cells and keratinocytes. Suprabasal keratinocytes immediately above dermal TNF-α-containing macrophages appear to produce IL-8, suggesting a cascade of cytokine expression involving several cell types. IFN-γ is a major cytokine released by activated T lymphocytes, and T lymphocytes are found at elevated levels in psoriatic skin [26]. Interestingly, elevated levels of IFN-γ have been reported in sera of patients with psoriasis [27], and patients with psoriasis injected with this cytokine to treat psoriatic arthropathy developed punctiform psoriatic foci at the injection site [28], suggesting a direct involvement of this cytokine in the pathogenesis of skin lesions in psoriasis. In contrast to TNF-α and IL-8, IFN-γ has not been found to be produced by keratinocytes (for review see [29]), although IFN-γ mRNA was detected in psoriatic epidermis using the sensitive reverse transcription polymerase chain reaction [30]. Taken together, these results suggest that in psoriatic skin IFN-γ may be produced by an epidermal cell type other than keratinocytes.

It should be noted that comparatively high levels of TNF-α (500 U/ml) and IFN-γ (1000 U/ml) were needed to induce significant changes of TGF-α and EGF/TGF-α receptor expression when compared to monolayer cultures [11]. However, the cytokine concentrations used here are comparable to the levels of IFN-γ (100 and 200 U/ml) required to induce ICAM-1 and HLA-DR on keratinocytes in situ [31]. By contrast to monolayer cultures, in the explant system access of cytokines to target cells is a critical determinant. Variables such as size of the skin explant, media composition, and modifications in technique may all influence diffusion of cytokines into the explant. In support of this notion, Griffiths et al [31] found that complete immersion of skin explants in culture medium leads to cytokine-induced expression of HLA-DR on keratinocytes, whereas partial immersion of primarily the dermal portion does not [15]. We chose to use the latter protocol based on the assumption that cytokines with possible relevance in vivo would gain access to keratinocytes by diffusion from the dermis up.

Of the other cytokines included in this study, IL-1 [32,33] and IL-6 [34] have been found at elevated levels or differently distributed in psoriatic lesions as compared to normal skin. Under the experimental conditions chosen in this study, these two cytokines did not affect TGF-α or TGF-α/EGF receptor expression detectably. However, the role of these cytokines in these phenomena cannot be discounted. Cytokine-induced effects could only be evaluated within a short time period (24 h) after initiation of explant culture. After this time period, cell viability decreased dramatically. It appears possible that some cytokines have indirect effects by triggering a sequence of events that require a longer time period to evolve. In support of this notion, IL-1 has been described to stimulate the production and release of IL-8 from keratinocytes [35,36].

**DISCUSSION**

In contrast to earlier studies on cultured keratinocytes, our experiments evaluated the cytokine-induced upregulation of TGF-α and its receptor on keratinocytes in situ. This approach takes into account that the net result of cytokine-mediated effects may be critically dependent on the complex interaction of different cell types and distinct differentiation states that are present in stratified skin, but absent in keratinocyte cultures.

The cytokine-induced expression pattern of immunoreactive EGF/TGF-α receptors in normal neonatal skin is strikingly similar to the pattern observed in active psoriatic lesions. In stratified normal epithelium, expression of immunoreactive EGF/TGF-α receptors is restricted to basal and immediate suprabasal keratinocytes ([21] and this study). However, in active psoriatic lesions, expression of EGF receptors is observed not only in the basal but also in the subcornal layers of stratified epithelium [6,22], reminiscent of the ubiquitous expression of EGF/TGF-α receptors in fetal skin [21]. In this study, TNF-α and IFN-γ treatment of skin explants produced a uniform expression of EGF/TGF-α receptors across all nucleated strata of the epidermis, similar to the pattern observed in psoriatic lesions or fetal epidermis.
Our results indicate that the pattern of TGF-α and EGF/TGF-α receptor expression in human epidermides can be modulated by cytokines. Cytokines known to be present in psoriatic lesions enhance the intensity and/or expand the distribution of TGF-α and its receptor in normal epidermides, causing it to resemble the psoriatic state. We hypothesize that the altered expression of TGF-α and its receptor observed in psoriatic lesions is secondary to an effect of a previously acting cytokine, either produced by infiltrating immune cells or the keratinocytes themselves.

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REFERENCES


