Human Keratinocytes Are a Source for Tumor Necrosis Factor α: Evidence for Synthesis and Release upon Stimulation with Endotoxin or Ultraviolet Light

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Summary

Tumor necrosis factor α (TNF-α), in addition to being cytotoxic for certain tumor cells, has turned out as a multifunctional cytokine that is involved in the regulation of immunity and inflammation. Since human keratinocytes have been demonstrated to be a potent source of various cytokines, it was investigated whether epidermal cells synthesize and release TNF-α. Supernatants derived from normal human keratinocytes (HNK) and human epidermoid carcinoma cell lines (KB, A431) were tested both in a TNF-α-specific ELISA and a bioassay. In supernatants of untreated epidermal cells, no or minimal TNF-α activity was found, while after stimulation with lipopolysaccharide (LPS) or ultraviolet (UV) light, significant amounts were detected. Western blot analysis using an antibody directed against human TNF-α revealed a molecular mass of 17 kD for keratinocyte-derived TNF-α. These biological and biochemical data were also confirmed by Northern blot analysis revealing mRNA specific for TNF-α in LPS- or ultraviolet B (UVB)-treated HNK and KB cells. In addition, increased TNF-α levels were detected in the serum obtained from human volunteers 12 and 24 h after a single total body UVB exposure, which caused a severe sunburn reaction. These findings indicate that keratinocytes upon stimulation are able to synthesize and release TNF-α, which may gain access to the circulation. Thus, TNF-α in concert with other epidermal cell–derived cytokines may mediate local and systemic inflammatory reactions during host defense against injurious events caused by microbial agents or UV irradiation.

TNF-α/cachectin has been originally described as a macrophage-derived factor that induces cachexia and hemorrhagic necrosis of tumors in animals, and exerts cytolytic effects on several tumor cell lines (1–3). In the meantime, it became evident that TNF-α exhibits a variety of activities, including release of prostaglandins and collagenase (4), and activation of neutrophils (5), eosinophils, and macrophages (6). Moreover, TNF-α increases MHC class I antigen (7) and intercellular adhesion molecule (ICAM)[1] expression on many cells (8), and induces the release of other cytokines. Through these multiple effects, TNF-α appears to be an essential mediator in inflammatory and immunologic reactions during host defense.

In addition to their barrier function, epidermal cells and, in particular, keratinocytes have been recently recognized to exhibit the capacity to release a variety of soluble mediators, including IL-1, IL-6, IL-8, CSF, transforming growth factors, and platelet-derived growth factor, that influence the growth, differentiation, and function of epidermal, dermal, and immunocompetent cells (9–11). The constitutive production of these factors usually is very low but can be induced significantly by various injurious agents, such as endotoxin, viral particles, tumor promotors, or UV light (9, 11). Consequently, these cytokines are not only involved in the mediation of local inflammatory reactions within the epidermis, but may also enter the circulation and thus cause systemic effects. Therefore, the present study was performed to investigate whether human keratinocytes in addition to these mediators can also release the multifunctional cytokine TNF-α/cachectin.

Materials and Methods

Cells and Cell Lines. Long-term cultures of normal human keratinocytes (HNK) were obtained from normal human foreskin as

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[1] *Abbreviations used in this paper:* GM-CSF, granulocyte/macrophage CSF; HNK, normal human keratinocytes; ICAM, intercellular adhesion molecule; LC, Langerhans cell; UVB, ultraviolet B.
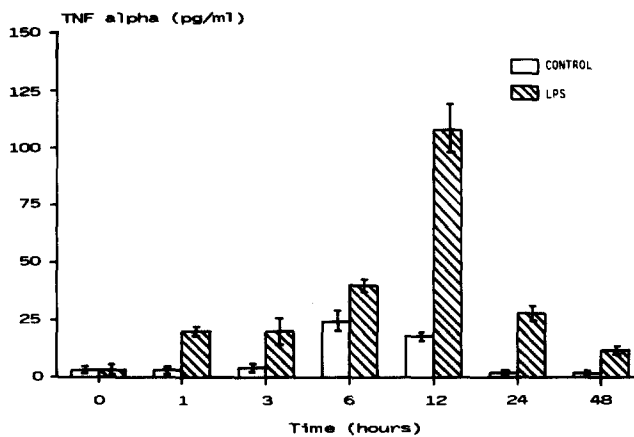
Figure 1. Human epidermoid carcinoma cell line cells (KB) release TNF-α upon stimulation with LPS (100 μg/ml). Supernatants of untreated or LPS-treated KB cells were harvested at different time points after stimulation and tested for TNF-α activity by the use of a TNF-α ELISA.
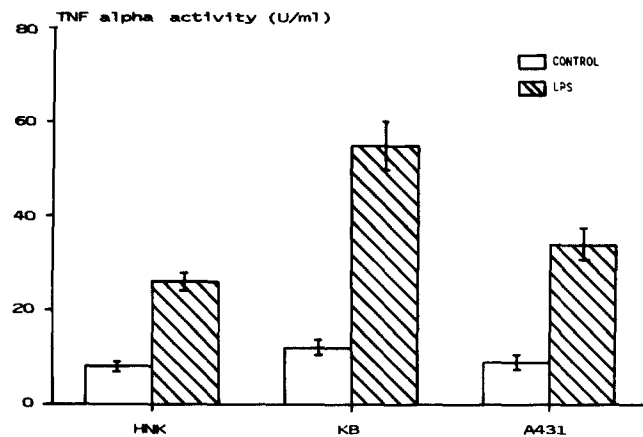

Figure 2. Epidermal cells release biologically active TNF-α. Supernatants of HNK, KB, or A431 cells either untreated or stimulated with LPS (100 μg/ml) were tested in the TNF-α-specific L-M bioassay. Culture supernatants were harvested 24 h after stimulation.

described previously and maintained in KGM (Clonetics, San Diego, CA) (12). Hydrocortisone was omitted from the culture medium 48 h before testing. The human epidermoid carcinoma cell lines KB and A431 (American Type Culture Collection, Rockville, MD) were maintained in monolayer cultures at 37°C in a humidified atmosphere containing 5% CO_2. To investigate factor production, cells were plated at a density of 5 × 10^5/ml in 24-well plates (Costar, Cambridge, MA), and supernatants were harvested at different time points (1, 3, 6, 12, 24, or 48 h), centrifuged at 1,000 g, and stored at −20°C. To stimulate cytokine production, cells were irradiated with ultraviolet B (UVB) light (FS20 bulbs; 10–200 J/m²) according to an established protocol (13) or cultured in the presence of LPS (100 μg/ml; Difco Laboratories, Detroit, MI).

In Vivo Exposure. After informed consent was obtained, four human volunteers were exposed to a single total body UVB exposure equivalent to four minimal erythema doses as previously described (14). As a light source, an unfiltered bank of six blue light lamps (Dr. Hoehnle, Munich, FRG) was used, emitting a linear spectrum between 250 and 600 nm with emission peaks at 300 and 360 nm, respectively. Blood was drawn from the antecubital vein into sterile heparinized tubes immediately before UV exposure, as well as 12 and 24 h after UV treatment.

Assay for TNF-α. TNF-α content of epidermal cell culture supernatants was assayed by determining the cytotoxicity of TNF-α against sensitive murine L-M cells (15). Briefly, L-M cells were seeded at a concentration of 4 × 10^4 cells/well in 100 μl culture medium and incubated for 22–24 h at 37°C in a 5% CO_2 atmosphere to establish a dense monolayer. 0.1 ml of culture supernatants to be tested was added in the presence of actinomycin D (2 μg/ml) for 18 h. Viability of cells was measured by staining with 0.5% crystal violet. After removal of excess dye and drying, absorbance was determined at 540 nm using a microplate reader (3550; Bio-Rad Laboratories, Richmond, CA).

In addition, a commercially available ELISA was used for measuring human TNF-α (Endogen, Boston, MA). This kit is based on the use of a mouse anti-TNF-α mAb and a rabbit polyvalent anti-TNF-α antibody. It is sensitive to 10 pg/ml TNF-α and does not crossreact with human lymphotoxin (TNF-β), IL-1, or IL-6.

Western Blot Analysis. Supernatants to be tested were concentrated 100 times by using Amicon ultrafiltration and were subjected to SDS-PAGE (15% total acrylamide concentration) under reducing conditions (16). After SDS-PAGE, proteins were electrotransferred to nitrocellulose and stained with a mouse mAb directed against recombinant human TNF-α (kindly provided by G. Adolf, Ernst-Boehringer Institute, Vienna, Austria). Biotinylated molecular weight markers were obtained from Sigma Chemical Co. (St. Louis, MO).

Northern Blot Analysis. Cultured cells were detached by treatment with ice-cold PBS and lysed in 4 M guanidine isothiocyanate. RNA was isolated by ultracentrifugation through cesium chloride, and subjected to electrophoresis in 1.0% agarose gel containing formaldehyde (2.2 M). After capillary transfer of RNA to nitrocellulose, membranes were prehybridized for at least 4 h with use of hybridizing solution containing 10% dextrane sulfate, 40% formamide, 20% 20× SSC, sonicated salmon sperm DNA (50 μg/ml), and yeast transfer RNA (50 μg/ml). TNF-α mRNA was detected by hybridization with a 0.8-kb EcoRI cDNA fragment (kindly provided by D. Pennica, Genentech, San Francisco, CA). After stringent washing conditions, blots were exposed to x-ray film at −70°C for 24 h (17).

Results

When epidermal cell–derived supernatants were evaluated for TNF-α production using a TNF-α-specific ELISA, no or only minimal amounts were found in conditioned medium obtained from unstimulated KB cells. However, when KB cells were treated with LPS (100 μg/ml), significant amounts of TNF-α were detected. TNF-α release started 1 h after stimulation and peaked at ∿12 h, returning to base line after 48 h (Fig. 1). Similar results were also obtained with HNK (data not shown). To test if the protein released by epidermal cells is biologically active, supernatants from HNK, A431, and KB cells were evaluated in the L-M bioassay. These supernatants again demonstrated only minimal amounts of active TNF-α until cells were treated with LPS (Fig. 2).

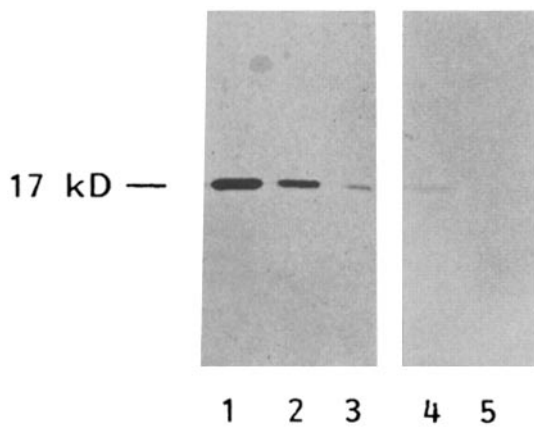To confirm that the cytotoxic activity against L-M cells observed in HNK supernatants is due to TNF-α, Western

Figure 3. Western blot analysis of keratinocyte-derived TNF-α. Supernatants derived from unstimulated (lane 5) or LPS (100 μg/ml)-treated HNK (lane 4) were harvested after 24 h and run on a 12% SDS gel. Staining was performed with a mouse mAb directed against recombinant human TNF-α. As a positive control, recombinant human TNF-α was used at different concentrations (100 ng, lane 1; 10 ng, lane 2; 1 ng, lane 3).

Figure 5. Stimulation of TNF-α production by UVB irradiation. HNK and KB cells were exposed to UVB light (100 J/m²). After a 24-h incubation, supernatants were tested for TNF-α using a TNF-α-specific ELISA. Results are expressed as pg/ml ± SD of three different experiments.

blot analysis using a mAb directed against human TNF-α was performed. A weak, but clearly detectable band with a molecular mass of 17 kD was observed in supernatants of LPS-stimulated HNK (Fig. 3).

To test whether the induction of TNF-α by epidermal cells is regulated at the transcriptional level, the expression of TNF-α mRNA in HNK and KB cells was examined. Unstimulated KB cells expressed little TNF-α mRNA (Fig. 4, lane 2), consistent with our previous biological data. However, treatment with LPS resulted in a marked increase in the level of TNF-α mRNA (Fig. 4, lane 1).

Since UVB light is well known to cause a significant dermal inflammatory response in vivo and is a potent inducer of cytokine production by epidermal cells in vitro as well (18), HNK and KB cells were exposed to UVB irradiation. In com-

parison with supernatants of untreated cells, both irradiated HNK and KB cells released significantly increased amounts of TNF-α (Fig. 5). Maximum production was observed when cells were exposed to 100 J/m², and supernatants were harvested after 24 h. In addition, UV-exposed HNK and KB expressed significantly increased TNF-α mRNA as evaluated by Northern blot analysis using a TNF-α-specific cDNA probe (Fig. 4, lane 3; Fig. 6). Maximum TNF-α expression was detected when RNA was extracted 12 h after UV treatment (Fig. 6).

After extensive solar exposure, increased levels of cytokines recently have been detected in the circulation (14, 18). Thus, serum samples from human healthy volunteers after total body UV irradiation causing severe sunburn reaction were evalu-
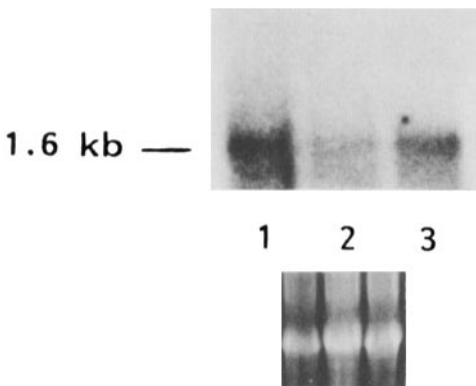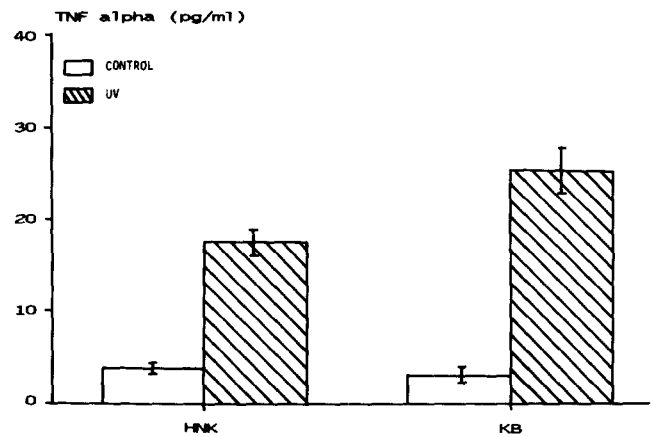

Figure 4. KB cells express mRNA encoding for TNF-α. KB cells were stimulated with LPS (100 μg/ml, lane 1), left untreated (lane 2), or irradiated with UVB (100 J/m², lane 3). After a 4-h incubation, RNA was extracted and hybridized with a cDNA probe encoding for TNF-α.
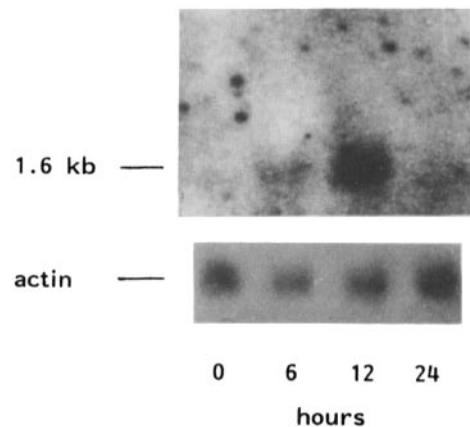

Figure 6. Northern blot analysis of UV-treated normal human keratinocytes. HNK cells were left untreated or irradiated with UVB light (100 J/m²), and total RNA was extracted at various time points and hybridized using ³²P-labeled TNF-α-specific cDNA. The blot was stripped and re-hybridized with a probe for β-actin (bottom) confirming that the RNA on the blot is intact, and that approximately equal amounts of RNA were loaded in each lane.
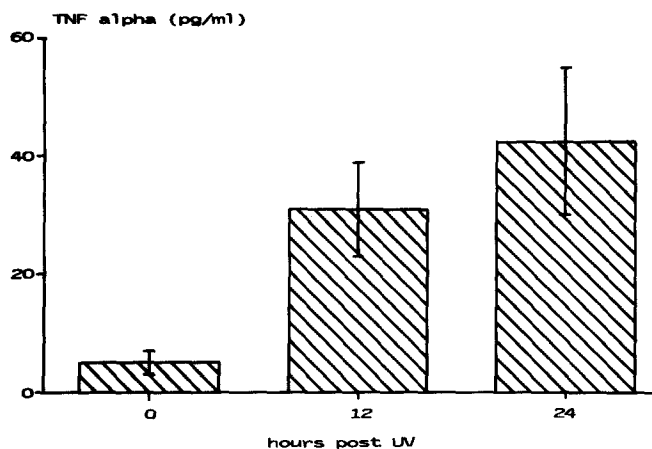
TNF alpha (pg/ml)

Figure 7. Serum TNF-α levels after UV treatment. Human healthy volunteers (n = 4) were treated with a single total body UVB exposure equivalent to four minimal erythema doses. Serum samples were obtained before as well as 12 and 24 h after UV irradiation. TNF-α was evaluated using the TNF-α-specific ELISA. Results are expressed as pg/ml mean ± SD.

ated for TNF-α using the TNF-α-specific ELISA. Serum samples obtained before UVB exposure contained minimal levels of TNF-α. However, significant circulating TNF-α was detected 12 and 24 h after UVB treatment (Fig. 7).

Discussion

The present study demonstrates that both HNK and the epidermoid carcinoma cell lines KB and A431 upon stimulation release immunoreactive TNF-α. This cytokine is produced as a biologically inactive prohormone that has to be cleaved at several sites to become the mature biologically active polypeptide (19, 20). Therefore, in addition to the ELISA, a TNF-α bioassay (L-M test) was utilized to prove that keratinocytes release a biologically active TNF-α. Accordingly, human keratinocytes produced low amounts of biologically active TNF-α. Nevertheless, it is hard to compare quantities of TNF-α measured with the ELISA with the quantity estimated in the bioassay, since one has to be aware of the fact that crude supernatants of keratinocytes and epidermoid carcinoma cell lines usually contain several other mediators that may interfere with bioassays (11). The identity of epidermal cell–derived TNF-α protein was confirmed by Western blot analysis using a specific mAb directed against TNF-α, which demonstrated a specific band with a molecular mass of 17 kD.

Keratinocytes constitutively secrete little TNF-α since TNF-α protein was hardly detectable in supernatants of untreated epidermal cells. However, upon stimulation with LPS or UVB, both HNK and epidermal carcinoma cells produced significant amounts of TNF-α. The results were also confirmed by Northern blot analysis detecting TNF-α mRNA in keratinocytes after stimulation with LPS or UVB. The present data clearly demonstrate that human keratinocytes upon stimulation can function as a source of TNF-α. The

quantities released, however, even after optimal induction, are much less than the amounts produced by macrophages.

Human keratinocytes have been demonstrated to release various cytokines, including IL-1α and β (21), IL-6 (22), IL-8 (23), CSF, transforming growth factors (11), and platelet-derived growth factor (10). Through the capacity to release these mediators, keratinocytes may play an important role in inflammatory reactions of the skin. Similar to the present findings concerning keratinocyte TNF-α release, the constitutive production of these factors both in vitro and in vivo is very low and has to be induced by various stimuli, including bacterial or viral products, tumor promotors, UV light, or cytokines themselves. TNF-α has been demonstrated to stimulate the release of other mediators, such as IL-1, IL-6, IL-8, granulocyte/macrophage (GM)-CSF, and platelet-derived growth factor in various cell types (24–27), and thus appears to be a key member of the cytokine cascade, playing an important role in pathologic events accompanying invasion of foreign organisms. Since the epidermis is frequently in contact with environmental microorganisms, it is not surprising that epidermal cells appear to be endowed with the capacity to release TNF-α. TNF-α functions in synergy with other cytokines, exhibiting some antiviral activity (28), and thus may be involved in the defense of the epidermis against viral particles. Moveover, TNF-α, like IL-1, increases ICAM-1 expression on fibroblasts, endothelial cells, and keratinocytes (8), resulting in an enhanced adhesion of lymphocytes, which may be important in certain lymphocyte-mediated skin diseases. The role of TNF-α as a regulatory cytokine in immunological as well as inflammatory processes is further supported by the finding that TNF-α enhances T and B cell functions, induces production of other cytokines, and modulates the activity of cytotoxic T cells (19).

An exaggerated release of TNF-α by the host can be toxic to the host, which is best demonstrated by the crucial role of TNF-α in the pathogenesis of endotoxic shock and cachexia in sepsis (29). TNF-α may also be involved in mediating the skin changes in GVHD. Accordingly, treatment of animals with anti-TNF antibodies prevents the development of cutaneous and intestinal lesions during the acute phase of GVHD (30). Recently, TNF-α has been detected in human epidermal cells of skin biopsies obtained from healthy subjects, and staining intensity was found increased after UVB exposure (31). Due to the use of only immunohistochemical techniques, this study did not definitely prove that keratinocytes are the real source of TNF-α, since it cannot be excluded that the cytokine is released by inflammatory cells in the dermis and just bound on the surface of keratinocytes. The results of our study, however, indicate that human keratinocytes appear to be the most likely source for TNF-α found within the epidermis.

According to the present data, UV light appears as an inducer of TNF-α production by epidermal cells in vitro. Since solar exposure can cause a significant inflammatory response in the skin, TNF-α may be involved in the mediation of this local reaction directly or via induction of other cytokines (24–27). In addition, this study demonstrates that after extensive UV exposure, increased TNF-α levels can be detected

in the circulation and thus may be responsible for systemic effects. Although keratinocytes are the primary target of UVB light and a source for TNF-α, it cannot be determined whether TNF-α found in the circulation is keratinocyte derived or not. Accordingly, cells other than keratinocytes (e.g., macrophages) may be considered as the source for enhanced circulating TNF-α, since in the present study, TNF-α serum levels continue to increase out to 24 h, whereas in vitro the message for TNF-α in keratinocytes has begun to decline at that time. UV exposure suppresses the immune response signficantly, e.g., blocks contact and delayed-type hypersensitivity reaction in mice (32). Recently, it has been demonstrated also that injection of TNF-α similar to exposure with UVB light inhibits the induction of contact hypersensitivity, suggesting that TNF-α released in enhanced amounts upon UV irradiation may be involved in the suppression of the immune response after UV exposure (33).

Currently, another remarkable function of TNF-α within the murine epidermis has been recognized. Murine epidermal Langerhans cells (LC) kept in culture mature into potent immunostimulatory dendritic cells (34). This maturation is mediated by two keratinocyte-derived cytokines: GM-CSF, which maintains LC viability and increases LC function; and IL-1, which in combination with GM-CSF enhances LC function (35). Recent studies indicate that LC cultured just in the presence of TNF-α had not matured as measured by their T cell stimulatory capacity, but still survived, suggesting that TNF-α serves as a crucial signal in LC viability (36). Accordingly, expression of TNF-α message has been detected in murine (BALB/c) epidermal cells (36).

In summary, the finding that human keratinocytes release TNF-α in addition to other cytokines further supports the crucial role of the epidermis, and in particular of the keratinocyte in the pathogenesis of both local and systemic inflammation after UV irradiation, as well as host defense against microbial organisms and tumors.

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