KERATINOCYTE GROWTH REGULATION BY THE PRODUCTS OF IMMUNE CELLS

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The induction of a delayed-type cell-mediated immune response in the skin of leprosy patients leads to extensive mononuclear cell accumulation in the dermis and alterations in the keratinocytes of the overlying epidermis (1). This includes keratinocyte proliferation, epidermal thickening, the expression of IFN-γ-induced peptide, IP-10 (2), and surface Ia antigen (1). Intradermal injection of human rIFN-γ into the skin of leprosy patients mimics the delayed cellular response to soluble antigen (3, 4). Not only are mononuclear leukocytes recruited into the dermal site, but proliferation and antigenic changes of the epidermal keratinocytes are also observed. In an effort to better understand the intercellular dialogues that occur in a cutaneous cell-mediated immune response, we have developed an in vitro assay to detect changes in keratinocyte multiplication in response to inflammatory cell supernatants and purified or recombinant cytokines. Evidence is presented that activated PBMC produce factors that modify keratinocyte growth and differentiation. Our results suggest that T lymphocytes are the cells responsible for this activity. We show that IFN-γ and transforming growth factor β (TGF-β)[1] inhibit keratinocyte growth, whereas IL-3 and granulocyte/monocyte colony-stimulating factor (GM-CSF) stimulate keratinocyte growth.

Materials and Methods

Human Keratinocyte Cultures. All experiments used normal human epidermal keratinocytes (NHEK; Clonectics Corp., San Diego, CA) at third passage. Cells were grown in modified MCDB 153 medium and keratinocyte growth medium (KGM) (Clonetics Corp.). KGM consists of MCDB 153 medium plus the added growth factors: epidermal growth factor (EGF, 10 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), and bovine pituitary extract protein (BPE, 70 μg/ml). Our basal medium was MCDB 153 medium and contained growth-limiting concentrations of hydrocortisone (10 ng/ml), insulin (100 ng/ml), and BPE (1.3 to 2.6 μg protein/ml). Second-passaged NHEK were grown in KGM on plastic 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY). Under these conditions, >96% of the keratinocytes were proliferating basal cells (5) and were free of fibroblast contamination (6). At 80–90% confluence, the keratinocytes were trypsinized (0.25% trypsin/0.02% EDTA; Gibco Laboratories, Grand Island, NY) to single cell suspensions, washed in MCDB 153, and seeded in 24-well plates (Costar, Cambridge, MA) for growth assays.

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[1] *Abbreviations used in this paper:* BPE, bovine pituitary extract protein; DC, dendritic cell; EGF, epidermal growth factor; FGF, fibroblast growth factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; KGM, keratinocyte growth medium; M-CSF, macrophage CSF; NHEK, normal human epidermal keratinocytes; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor β.

In Vitro Keratinocyte Growth Assay. Keratinocytes were seeded at 5×10^3 to 10^4 keratinocytes per well in KGM. After overnight culture (37°C, 5% CO_2) the nonadherent keratinocytes were aspirated and 1 ml MCDB 153 medium was added. Plating efficiency was usually 30%. At 48–72 h of culture, the MCDB 153 medium was replaced with 1 ml of fresh basal medium with or without increasing concentrations of purified or recombinant cytokine, growth factor, or inflammatory cell supernatant, and cultured for 48–96 h. Cell counts were performed on triplicate trypsinized cultures using a Coulter Counter (Model ZF; Coulter Electronics, Hialeah, FL). Cell viability was evaluated by Trypan Blue exclusion (0.4%, Sigma Chemical Co., St. Louis, MO) and was >95%.

Cytokines and Growth Factors. EGF, insulin, hydrocortisone, and BPE were obtained from Clonetics Corp.; purified human TGF-β, human rIL-1β, human rIL-2, and purified human platelet-derived growth factor (PDGF) from Collaborative Research (Bedford, MA); human rIL-3, macrophage colony-stimulating factor (M-CSF), and GM-CSF from Genetics Institute (Cambridge, MA); purified human fibroblast growth factor (FGF [basic]) from Rafael Mira y Lopez (The Rockefeller University), and human rIFN-γ from Genentech (San Francisco, CA).

Con A-stimulated PBMC Supernatants. Human PBMC (platelet-free, reference 7) obtained from buffy coats (New York Blood Center, New York, NY) were separated on Ficoll-pacque (Pharmacia Fine Chemicals, Piscataway, NJ), washed, and cultured (2×10^6/ml) for 0–96 h in serum-free MCDB 153 medium containing 1 μg Con A/ml (Pharmacia). Harvested supernatants were clarified by centrifugation (500 *g*), stored at −20°C, and freshly thawed before use.

Supernatants from Mixed Leukocyte Reactions (MLR). Allo-MLR supernatants were obtained by using monocyte or dendritic cell (DC) and T lymphocyte enriched populations (8). Briefly, 3×10^5 irradiated (3,000 rad, ^{137}Cs) monocytes or DC were incubated 7 d with 10^7 allogeneic responding T cells in 10 ml RPMI 1640 (Gibco Laboratories) supplemented with 100 U penicillin/ml, 100 μg streptomycin/ml, 2 mM glutamine, and 10% AB^+ human serum. Supernatants were clarified by centrifugation (500 *g*) and stored at −20°C until use.

Results

Growth Assay. Measurement of human keratinocyte growth in vitro generally is determined by an increase in colony number or area after 2 wk of culture in 60-mm dishes (9–11). Such assays use primary isolated keratinocytes potentially contaminated with dermal fibroblasts or use serum in addition to irradiated fibroblast feeder layers. Thus analysis of the direct effect of an added putative growth factor on keratinocyte replication is difficult. Others have used thymidine uptake as an indicator of proliferation (12), but this has had no correlation with in vitro keratinocyte growth (13) as human keratinocytes have been shown to catabolize thymidine (14). To determine the effects that factor(s) generated locally during a cutaneous cell-mediated response have on keratinocyte replication, we have developed an assay that uses primary human keratinocytes free of fibroblasts, feeder layers, and cultured in serum-free medium. This system allows us to measure keratinocyte multiplication by changes in cell number. In contrast to other bioassays, this yields results within 72–96 h.

Keratinocyte Growth in KGM vs. MCDB 153. Fig. 1 shows the kinetics of keratinocyte replication in complete (KGM) medium (with added growth factors) compared with that observed in basal MCDB 153 medium alone. 48 h after culture, keratinocyte growth was greater in KGM than in MCDB 153 medium. By 96 h, this difference was accentuated as cells cultured in MCDB 153 medium ceased to divide. Although keratinocytes failed to divide in MCDB 153 medium, they were still viable and could be stimulated to multiply if growth factors were added to the medium (data not shown). Thus the range available to measure stimulatory/inhibitory factor(s) on keratinocyte growth is from ~$6–18 \times 10^4$/well at 96 h of culture.

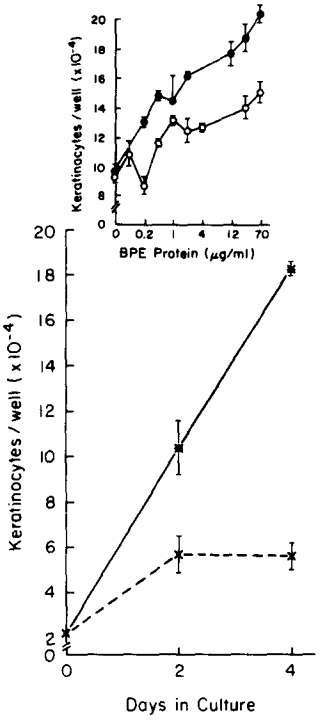

FIGURE 1. In vitro keratinocyte replication. Human keratinocytes were cultured for 96 h either in KGM (*) or MCDB 153 medium (x) alone. (*Inset*) Keratinocytes were cultured with increasing concentrations of BPE protein/ml MCDB 153 medium (100 ng insulin/ml and 10 ng hydrocortisone/ml) for 48 (○) and 72 (●) hours. Results are expressed as the mean of triplicate wells ± 1 SEM.

To detect inhibitory and/or stimulatory molecules generated during a local inflammatory response, it was necessary to modify the medium such that it would maintain a low level of keratinocyte proliferation. In our assay hydrocortisone and insulin (10 ng/ml and 100 ng/ml MCDB 153, respectively) allow keratinocyte multiplication (not shown). EGF was not mitogenic in our assay as reported by others (15, 16). Fig. 1 (*inset*) demonstrates the effect of increasing concentrations of BPE protein on keratinocytes in the presence of hydrocortisone and insulin. At 48 h, the number of keratinocytes recovered from wells containing 70 μg BPE protein/ml medium was almost double that of wells without BPE protein. After 72 h of culture this difference was nearly threefold. Consequently, growth experiments used a basal medium that contained MCDB 153 medium with hydrocortisone (10 ng/ml), insulin (100 ng/ml), and BPE protein (1.3–2.6 μg/ml) and resulted in an approximate doubling in cell number every 48 h.

Keratinocyte Growth Factors Generated by Activated Immune Cells. Fig. 2 shows the regulatory factors produced in vitro by human PBMC stimulated for 24–72 h with or without 1 μg Con A/ml MCDB 153 medium. Culture of keratinocytes for 72 h in 33% Con A–stimulated PBMC supernatants inhibited cell growth (Fig. 2 *A*) as compared with supernatants from unstimulated PBMC that possess stimulatory properties at this concentration (Fig. 2 *B*). In fact, the activity found in the control PBMC supernatants was as stimulatory as complete medium. Stimulatory activity (45%) was also observed with a low concentration (0.01%) of Con A–stimulated PBMC supernatant. Con A itself did not have suppressive or stimulatory effects (Fig. 2 *C*). Thus, both Con A–stimulated and nonstimulated cell supernatants contain stimulatory

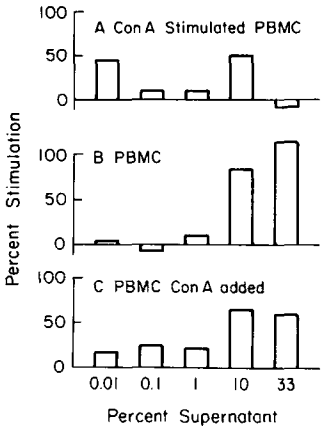

FIGURE 2. The effect of human PBMC supernatants on in vitro keratinocyte growth. Supernatants were obtained from human PBMC cultured with (*A*) or without (*B, C*) 1 μg Con A/ml MCDB 153 medium from 24 to 72 h. Percent stimulation of keratinocyte growth above that obtained with basal medium alone (abscissa) was determined after 72 h of culture. The average of two such experiments is shown. (*C*) Addition of 1 μg Con A/ml to supernatants from PBMC not activated with Con A. [^{3}H]TdR incorporation for PBMC stimulated with Con A (1.8×10^3 cpm) were 10-fold greater than nonstimulated cultures (0.1×10^3 cpm).

factors for keratinocyte replication, but at different effective concentrations (0.01% and 10%, respectively).

The Effect of Human Allo-MLR Supernatants on Keratinocyte Growth. To identify the mononuclear cell responsible for the keratinocyte growth factors found in PBMC supernatants, allo-MLR supernatants were generated. Fig. 3 shows data in which supernatants from bulk PBMC, plastic adherent monocytes, and T lymphocytes stimulated with either irradiated monocytes or DC were examined for their stimulatory properties after 48 h of culture. To examine this property in the absence of serum inhibitory factors, all supernatants were diluted to 1% of culture medium. At this concentration very little effect was observed in supernatants from nonstimulated monocytes, PBMC, or basal medium. In contrast, both 7-d MLR supernatants contained molecule(s) capable of stimulating keratinocyte replication. The percent stimulation of DC-activated T lymphocytes was almost twice that of monocyte-activated T cells. This suggests that T lymphocytes, rather than monocytes are the cells producing and releasing keratinocyte growth-promoting molecules.

Keratinocyte Replication After Culture with Recombinant or Highly Purified Cytokines and Growth Factors. Cytokines that might be produced in the in situ environment and modify keratinocyte growth were examined. This revealed that GM-CSF and IL-3 possessed stimulatory properties (Fig. 4, *A* and *B*), while TGF-β and IFN-γ were potent inhibitors of keratinocyte replication (Fig. 4, *C* and *D*). Peak stimulation with GM-CSF occurred at 0.6 ng/ml basal medium (Fig. 4 *A*), whereas the peak stimulatory activity for IL-3 occurred at 2.5 ng/ml basal medium (Fig. 4 *B*). The stimulatory effect of IL-3 was enhanced by adding fresh lymphokine at 24-h intervals.

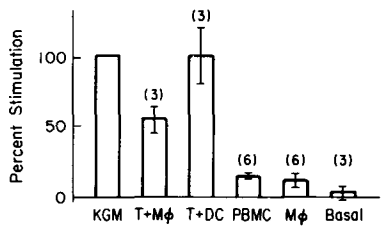

FIGURE 3. Keratinocyte growth after culture in allo-MLR supernatants. The figure represents the effects of 1% supernatants from allo-MLRs, nonactivated human PBMC, plastic adherent monocytes, and basal medium alone on keratinocyte growth at 48 h of culture. Percent stimulation was compared with that obtained after culture in KGM. Nonactivated monocyte and PBMC supernatants were obtained from 48- and 72-h cultures, respectively. Allo-MLR supernatants were obtained after 7 d of culture. The numbers in parentheses equal the number of experiments performed.

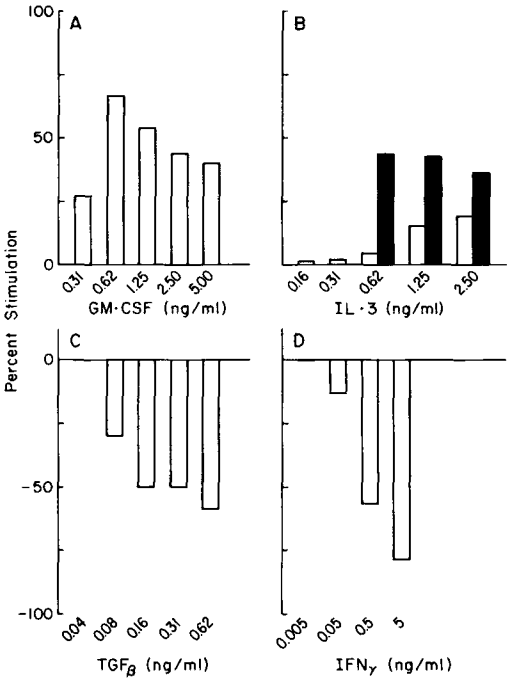

FIGURE 4. The effect of purified or recombinant cytokines and growth factors on keratinocyte growth. Keratinocytes cultured in 1 ml of fresh basal medium were fed on day 0 only (*open bars*) or at 24-h intervals (*closed bars*) with increasing concentrations of GM-CSF (*A*), IL-3 (*B*), TGF-β (*C*), or IFN-γ (*D*). Percent stimulation was determined at 72 (*D*) or 96 (*A, B, C*) h of culture and compared with that obtained with basal medium alone (abscissa).

Our observations demonstrated that keratinocyte replication was inhibited at concentrations of TGF-β as low as 0.04–0.08 ng/ml basal medium (Fig. 4 *C*). The inhibition was fully reversed when the cells were washed free of cytokine and new growth medium was added (data not shown). Greater than 0.05 ng IFN-γ/ml basal medium was strongly inhibitory (Fig. 4 *D*), and washing the cultures free of IFN-γ and the addition of new growth medium demonstrated that the inhibitory effect was concentration dependent and not completely reversible (data not shown). This agrees with previously published reports (11, 17). Treatment of the cells with 1 ng/ml or more IFN-γ induced the expression of class II molecules and suggests the terminal differentiation of a subpopulation of keratinocytes. Several other cytokines and growth factors, including EGF, PDGF, FGF (basic), IL-1, IL-2, and M-CSF were not stimulatory in this system (Table I). However, PDGF and IL-1 were suppressive for keratinocyte growth at high concentrations.

Discussion

We have previously shown that the generation of a delayed-type immune response in the human dermis leads to the growth and differentiation of the overlying epidermis (1). Detailed examination of this process is now possible through the development of an in vitro assay for the growth of purified keratinocytes. In short-term assays, we have tested the molecules secreted by T cells and monocytes and compared their growth stimulating and inhibiting properties with those of pure, recombinant cytokines.

Clear-cut growth stimulation occurred with purified T cells cultured with DC as accessory cells. At 1% concentration of supernatant, this proved as effective as the

TABLE I

The Effect of Various Cytokines and Growth Factors on In Vitro Keratinocyte Proliferation

Cytokine/growth factor	Range tested*	Mitogenic effect
IL-1β	0.7–700 pg/ml	None (inhibition at 700 pg/ml)
IL-2	0.002–6.8 ng/ml	None
M-CSF	0.3–5.0 ng/ml	None
EGF	0.01–100 ng/ml	None
FGF (basic)	0.01–45 ng/ml	None
PDGF	0.6–2.5 ng/ml	None (inhibition at 2.5 ng/ml)

* Cytokines/growth factors were tested for their effects at 24, 48, 72, and 96 h of culture.

complete keratinocyte growth medium. The nature of factors in T cell medium might include a variety of cytokines, some of which have recently been cloned. Addition of IL-2, M-CSF, TGF-β, or IFN-γ either led to no effect on growth or in the case of TGF-β and IFN-γ, marked inhibition of growth at both high and low concentrations. This was somewhat suprising in that the direct injection of human rIFN-γ into the skin leads to enhanced keratinocyte proliferation and thickening of the epidermis (3, 4). We suspect, therefore, that IFN-γ administrated in vivo promotes the production of growth factors by other cells of the skin.

Two recombinant lymphokines proved to stimulate keratinocyte growth (IL-3 and GM-CSF). These novel findings suggest that IL-3 may regulate in vitro human keratinocyte growth. The growth response to IL-3 is saturable at low concentrations and suggests that keratinocytes possess a high affinity IL-3-R. The local production of IL-3 by activated, dermal T cells may therefore regulate epidermal growth. GM-CSF is also a molecule of some interest since it is synthesized by keratinocytes and has the ability to capacitate freshly isolated murine Langerhans' cells for their accessory function (18). It is therefore possible that in the presence of T cell–derived lymphokines, keratinocytes may themselves secrete GM-CSF, and that this molecule serves as an autocrine growth regulator.

T cells and their secretory repertoire may serve to regulate the epidermis in both physiological and pathological situations. During a short-term antigen-driven event, keratinocyte growth, epidermal thickening, and class II gene expression would serve to enhance wound healing, antigen presentation, and the barrier function of the skin. These T cell–derived molecules would help to amplify cell-mediated immunity by recruiting keratinocytes as active participants. In contrast, the chronicity of diseases such as psoriasis (19) and its associated T cell infiltrates could have adverse effects on skin integrity.

Dissection of the comparative contributions of T cells and monocytes was made possible by the use of purified DC as accessory cells (8). It was clear that monocyte culture supernatants contained inhibitory molecules, whereas T cell supernatants gave growth stimulatory effects. Monocyte culture fluids may contain a number of potentially inhibitory molecules. These include arachidonic acid metabolites such as prostaglandin E_2 and TNF (7, 20). Both are secreted in large amounts after the

uptake of particulates and exposure to LPS endotoxin (reviewed in reference 21). Monocyte supernatants also have been shown to contain growth stimulants such as IL-1, PDGF, fibroblast growth factor, and fibroblast TGF-β (21). Their stimulatory activity on keratinocytes, if they exist, may be masked by the presence of the previously described inhibitors.

Summary

We describe a bioassay that allows the in vitro investigation of the stimulatory and suppressive factors derived from immune cells in short-term cultures of human keratinocytes. In agreement with other assays, epidermal growth factor is not mitogenic for human keratinocytes. Supernatant fluid from human PBMC stimulated with Con A, from allo-MLRs, as well as supernatants from nonstimulated PBMC, possess growth-promoting molecules. Our results show that both activated and nonactivated T cells release growth factors. Suppressive molecules are produced preferentially by monocyte cultures. Two T cell products, IFN-γ and transforming growth factor β are both inhibitory for keratinocyte proliferation. Two other T cell products, IL-3 and GM-CSF, stimulate keratinocyte proliferation at nanogram concentrations. These results suggest the existence of regulatory circuits between the T cells of a dermal inflammatory infiltrate and the overlying epidermal keratinocytes. This may determine the fine control of epidermal proliferation and turnover leading either to enhanced wound repair or skin pathology.

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