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Authors

Halaban, R
Langdon, R
Birchall, N
et al.

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Basic Fibroblast Growth Factor from Human Keratinocytes Is a Natural Mitogen for Melanocytes

R. Halaban, R. Langdon, N. Birchall, C. Cuono,* A. Baird,‡ G. Scott, G. Moellmann, and J. McGuire

Departments of Dermatology and *Plastic and Reconstructive Surgery, Yale University School of Medicine, New Haven, Connecticut 06510; and ‡Department of Neuroendocrinology, Salk Institute for Biological Studies, La Jolla, California 92037

Abstract. To survive and proliferate in pure culture, human melanocytes require basic fibroblast growth factor (bFGF) and cAMP. Without these factors, even in the presence of serum, the cells die. Melanocytes cultured in the presence of keratinocytes, however, survive for weeks without added bFGF and cAMP. We show here that the growth factor for melanocytes produced by human keratinocytes is bFGF because its activity can be abolished by neutralizing antibodies to bFGF and by a bFGF synthetic peptide that inhibits the binding of the growth factor to its receptor. The melanocyte mitogen in keratinocytes is cell associated and increases after irradiation with ultraviolet B. Northern blots reveal bFGF gene transcripts in keratinocytes but not melanocytes. These studies demonstrate that bFGF elaborated by keratinocytes in vitro sustains melanocyte growth and survival, and they suggest that keratinocyte-derived bFGF is the natural growth factor for normal human melanocytes in vivo.

EPIDERMAL melanocytes survive and may even proliferate for several weeks when cultured in the presence of keratinocytes (Prunieras et al., 1964). In contrast, melanocytes die within a week when cultured alone in routine media (Halaban, 1988) or medium optimal for keratinocyte proliferation (our unpublished results), because melanocytes require two classes of mitogen. One class is represented by TPA (12-*O*-tetradecanoyl phorbol-13-acetate), the other by substances such as cholera toxin, isobutylmethyl xanthine (IBMX),[1] or dibutyryl cAMP (dbcAMP), which increase intracellular cAMP (Eisinger and Marko, 1982; Halaban et al., 1986). Recently, we have shown that basic fibroblast growth factor (bFGF) can substitute for TPA and is the melanocyte mitogen in extracts of human melanomas and placenta, bovine pituitary gland and other bovine organs (Halaban et al., 1987). Since, in situ, normal human melanocytes are surrounded by basal keratinocytes and in culture can be sustained by contact with keratinocytes, we asked whether keratinocytes might stimulate melanocytes via bFGF. The studies described here demonstrate that proliferating human keratinocytes in culture contain a mitogen for human melanocytes, which increases up to sixfold after ultraviolet B (UVB) irradiation. This mitogen is bFGF because the mitogenic activity of keratinocyte extract is inhibited by neutralizing antibodies to bFGF and also by a synthetic peptide that blocks the binding of bFGF to its receptor. Northern blot analysis with a bovine cDNA probe for bFGF reveals two species of bFGF gene transcripts in rapidly proliferating keratinocytes. The sizes of these transcripts match those characteristically found with this probe in tissues and cultured cells that are known to synthesize bFGF. These findings suggest that in situ the viability of the epidermal melanocyte population is regulated by bFGF from basal, proliferative keratinocytes.

1. *Abbreviations used in this paper*: bFGF, basic fibroblast growth factor; dbcAMP, dibutyryl cAMP; IBMX, isobutylmethyl xanthine; UVB, ultraviolet B.

Materials and Methods

Cell Culture

Normal human melanocyte cultures were initiated from neonatal foreskins in TIP medium, consisting of 85 nM TPA (LC Services Corp., Wolburn, MA), 0.1 mM IBMX (Sigma Chemical Co., St. Louis, MO), and 10–20 μg protein/ml placental extract in Ham's F-10 medium (American Biorganics, Inc., N. Tonawanda, NY) that was supplemented with 10% newborn calf serum (Gibco Laboratories, Grand Island, NY), 200 U/ml penicillin, 100 μg/ml streptomycin (Halaban et al., 1986). Where indicated, melanocytes were grown in TIC medium in which 2.5 nM cholera toxin was substituted for placental extract. Contaminating fibroblasts were eliminated by incubating the cultures for 3–4 d in TIP medium supplemented with 100 μg/ml geneticin (G418 sulfate; Gibco Laboratories; Halaban and Alfano, 1984). The melanocytes used in the experiments had been in culture for no longer than 4 mo and had been passed no more than five times at a ratio of 1:3.

Keratinocyte cultures were initiated from neonatal foreskins and adult human skin on collagen-coated Petri dishes in modified MCDB-153 (a low calcium medium containing 0.03 mM $CaCl_2$; Irvine Scientific, Santa Ana, CA), supplemented with antibiotics as described above, 0.63 μg/ml fungizone (Flow Laboratories, Inc., McLean, VA), 70 μg protein/ml bovine pituitary extract (Pel-Freeze Biologicals, Rogers, AR; prepared as described by Halaban et al., 1987), 1 ng/ml epidermal growth factor (Sigma Chemical Co.), 50 μM hydrocortisone (Gibco Laboratories), 0.1 mM ethanolamine, and 0.1 mM phosphoethanolamine (Sigma Chemical Co.). Subsequently, keratinocytes were passed twice in the same medium in uncoated culture flasks. Adult keratinocytes were derived from uninjured skin of burn pa-

tients or from cadavers. Where indicated, the cells were maintained in DME (containing 3.0 mM $CaCl_2$; Gibco Laboratories) supplemented with 20% FCS (HyClone Laboratories, Logan, UT) in which keratinocytes stratify. To test the effect of interleukin 1, keratinocytes were grown in MCDB-153 medium and incubated for 24 h with 0.02 μg/ml human recombinant interleukin 1 (Hoffman-LaRoche, Inc., Nutley, NJ).

Fibroblasts from neonatal foreskins were grown in DME supplemented with 10% calf serum and were used after the second or third passage. SK-HEP-1, a human hepatoma cell line, and 3T3-Swiss murine fibroblasts (ATCC CCL 92) were grown in the same medium.

To compare the viability of melanocytes with and without keratinocytes, melanocytes obtained from the foreskin of a black baby were seeded onto (*a*) confluent cultures of allogeneic neonatal keratinocytes; (*b*) into wells in which approximately half the surface was occupied by keratinocytes; and (*c*) into wells without keratinocytes. Co-cultures were set up also with melanocytes and dermal fibroblasts. The cultures received MCDB-153, DME, or TIP medium; these media support, respectively, keratinocyte proliferation, keratinocyte stratification, and melanocyte proliferation.

UVB Irradiation

Cells were irradiated with 150 mJ/cm² of UVB light (wavelength 290–310 nm) from a panel of four lamps (model FS20 Sun Lamp; Westinghouse Electric Corp., Pittsburgh, PA) at 1.5 mW/cm². Incident dose at the cell surface was measured through one layer each of tissue culture plastic and medium by means of a UVX digital radiometer (Ultra-Violet Products, Inc., San Gabriel, CA; Kupper et al., 1987). Keratinocytes and fibroblasts, grown in 150 cm⁵ flasks, were collected 7–9 and 24 h after UVB irradiation. Extracts of these cells wee used to test for mitogenic activity toward melanocytes.

Melanocytes were seeded in 8-cm² Petri dishes (16,000 cells/cm²) and incubated in serum-supplemented Ham's F-10 medium containing TPA, IBMX, and cholera toxin (TIC); or TPA alone; or IBMX and cholera toxin without TPA or without any growth factor. The following day, 27 and 47 h later, sets of two dishes from each culture condition were irradiated with 50 mJ/cm² of UVB light. At various intervals thereafter, DNA synthetic activity was measured for 1 h by the [³H]thymidine incorporation assay described below. Nonirradiated cells served as controls.

Tyrosinase activity of irradiated and nonirradiated melanocytes was measured in cell extracts as described (Halaban et al., 1983). A unit of tyrosinase was defined as the activity of enzyme that catalyzed the oxidation of 1 μmol of tyrosine in 1 min.

Preparation of Cell Extracts, Antibodies, and Assay for Mitogenic Activity toward Melanocytes

To prepare extracts, cells were scraped off the culture surface, suspended in PBS, centrifuged, and washed two times with PBS. The cell pellets were resuspended in 0.2–0.5 ml PBS or double-distilled water and sonicated on ice. Alternatively, cells were disrupted by three cycles of freeze-thawing. Mitogenic activity of the extracts was similar, regardless of the procedure used. The disrupted cells were centrifuged at 13,500 *g* for 10 min at 4°C, and 5-μl aliquots of the supernatants were taken for protein determination by the Bio-Rad assay (Bio-Rad Laboratories, Cambridge, MA). BSA served as a control. To test the mitogenic activity of cell extracts toward melanocytes, melanocyte cultures, 24,000–80,000 cells/4-cm² well (Costar Data Packaging Corp., Cambridge, MA), were incubated overnight in PC-1 defined medium (Ventrex Laboratories, Inc., Portland, ME) without serum and without added growth factors (Halaban et al., 1987). This medium was then removed and experimental media (1 ml per well) were added.

Control serum and antiserum (Halaban et al., 1987) were used as such or stirred at room temperature with 18% Na_2SO_4 for 1 h and then centrifuged for 10 min at 17,000 *g*. Precipitate was resuspended in H_2O at half original volume, diluted 1:1 with PC-1 medium, and passed through a 0.22-μm Millex-GV filter (Millipore Continental Water Systems, Bedford, MA). This preparation is referred to as immunoglobulin fraction.

Experimental media were prepared by adding known amounts of cell extract to PC-1 medium which in some experiments was then passed through a 0.22-μm Millex-GV filter. The mitogenic activity was not affected by filtration. Stimulation of melanocyte growth by the extracts was assayed at the end of 24 or 48 h by determining [³H]thymidine incorporation as a measure of DNA synthesis. For this purpose, the experimental media were exchanged with assay medium consisting of minimal essential medium without calcium and magnesium (MEMS; Gibco Laboratories), and containing 5 μCi/ml [³H]thymidine (90 Ci/mmole, 0.5 ml/well; Amersham Corp., Arlington Heights, IL). At the end of 1–3 h incubation, 0.3 ml of trypsin-EDTA solution in MEMS was added to detach the cells from the culture dishes. The detached cells were trapped onto No. 30 glass filters in the Minifold apparatus of Schleicher & Schuell, Inc. (Keene, NH; American Bioanalytical, Natic, MA). The filters were washed four times with distilled water, dried, and placed in scintillation fluid. Radioactivity was determined in a scintillation counter.

Northern Blot Analysis

RNA was extracted by the procedure of Chomczynski and Sacchi (1987). Total or polyadenylated RNAs were fractionated on 1.5% agarose denaturing gels containing formaldehyde and transferred to nitrocellulose filters. The blots were pretreated with BLOTTO buffer, containing 50% formamide and hybridized in the same buffer, supplemented with 10% dextran sulfate (Siegel and Bresnick, 1986) and ³²P-labeled 1.4-kb Eco RI fragment of cDNA for bFGF. The cDNA fragment (for bovine bFGF in pBR322 plasmid, known also as pJJ11-1; Abraham et al., 1986*a*) was obtained from Drs. J. A. Abraham and J. C. Fiddes, California Biotechnology, Inc., Mountain View, CA.

Results

Evidence that Keratinocytes Synthesize a Mitogen toward Melanocytes

The light microscopic appearance of melanocytes in MCDB-153 medium with and without keratinocytes is shown in Fig. 1. In mixed culture, those melanocytes that were in direct contact with a keratinocyte survived for >2 wk and sprouted dendrites toward neighboring keratinocytes. In pure culture and in mixed cultures in which keratinocytes were sparse, the melanocytes became spindle shaped then rounded up, and lost their viability after 1 wk. Loss of viability was defined as an inability to incorporate [³H]thymidine 1 d after restimulation with TPA and IBMX (Halaban, 1988). Conditioned medium from keratinocyte cultures in which melanocytes survived did not support proliferation or survival of pure cultures of melanocytes (Table I). The slight stimulation at low dilution of conditioned medium may have been due to mitogen released from lysed keratinocytes. These results indicate that direct contact of melanocytes with keratinocytes supports the viability of melanocytes and suggests that the growth factor for melanocytes, produced by keratinocytes, is not secreted freely into the medium. Keratinocytes were more effective than fibroblasts in supporting the survival of melanocytes because fibroblasts outgrew and hence overcrowded and displaced the melanocytes (data not shown).

Mitogenic activity toward melanocytes in extracts of keratinocytes is shown in Table I and Fig. 2. The data demonstrate that keratinocyte extract without addition of cAMP stimulated DNA synthesis in melanocytes to some extent and that stimulation was enhanced 10–20-fold by 1 mM dbcAMP. MCDB-153 medium, optimal for keratinocyte proliferation, was not a requirement in the melanocytic response. In fact, PC-1 medium, which promotes optimal growth of most other cells, was also more conducive to the proliferation of melanocytes (Table I). Nevertheless, the medium in which the keratinocytes were maintained affected the level of melanocyte mitogen in the extracts. As shown in Fig. 2, extract from keratinocytes grown in serum-free, low calcium medium (MCDB-153), optimal for keratinocyte proliferation, had ~20-fold higher mitogenic activity toward melanocytes than a similar amount of extract from keratinocytes grown in DME, containing serum and high calcium, optimal for keratinocyte stratification. The mitogenic dose response of melanocytes to keratinocyte extract was biphasic, similar to the

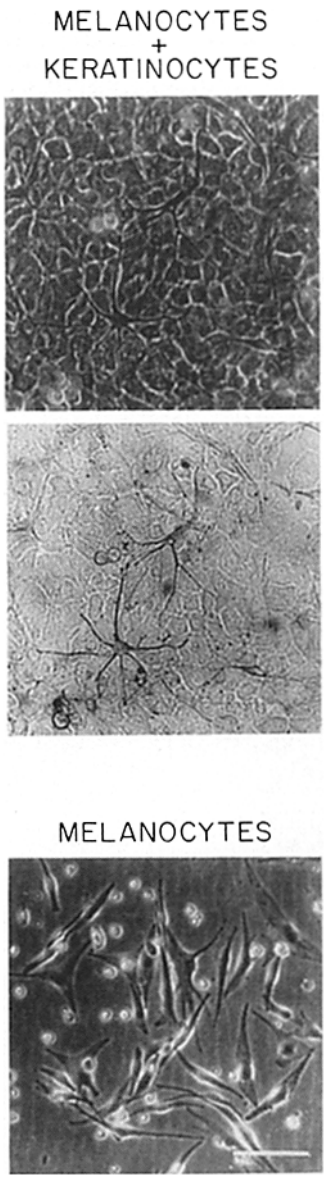

Figure 1. Morphology of melanocytes cultured with and without keratinocytes. (*Top*) Phase-contrast and bright field photomicrographs, respectively, of human keratinocytes and melanocytes cultivated together in MCDB-153 medium for 2 wk. Melanocytes are viable and remain highly dendritic. (*Bottom*) Phase-contrast micrograph of a pure culture of melanocytes grown in MCDB-153 medium for 5 d. The rounded cells are dying melanocytes. Bar, 112 μm.

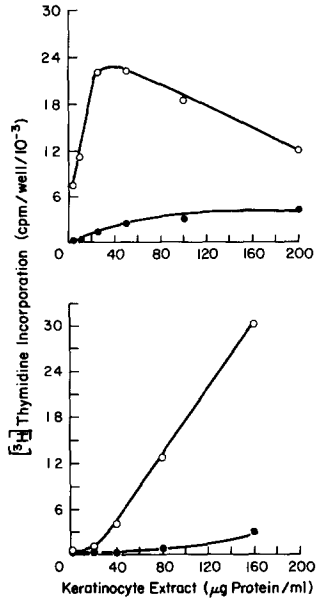

Figure 2. Dose-related mitogenic stimulation of human melanocytes by extracts from human keratinocytes. Melanocytes, derived from newborn foreskin, were grown in Ham's F-10 medium supplemented with TPA, IBMX, and placental extract (TIP) and seeded without TIP into 4-cm² wells in PC-1 medium 1 d before the addition of keratinocyte extract. Extracts were added with (○) or without (●) 1 mM dbcAMP. Melanocytes treated with dbcAMP but without cell extract did not incorporate a significant amount of [³H]thymidine (~100 cpm/well). [³H]thymidine incorporation into melanocytes was measured over the final 2–3 h of a 24-h incubation with experimental media. Values are averages of cpm from two wells per 1 h. (*Top*) Extract was prepared from proliferating keratinocytes derived from newborn foreskin and grown in MCDB-153 medium for a month. (*Bottom*) Extract was prepared from stratifying keratinocytes, derived from adult skin, propagated in MCDB-153 medium for 2 wk, and maintained thereafter in DME for 10 d to induce stratification. The experiments presented in the top and bottom figures were carried out at different times. Due to variations in [³H]thymidine incorporation between cultures, the kinetics of the response should be compared and not the cpm values.

dose response to bFGF (Halaban et al., 1987) in that keratinocyte extracts given at concentrations higher than those required for optimal melanocyte proliferation elicited only a suboptimal mitogenic response from melanocytes. The optimal concentration of extract from keratinocytes grown in MCDB-153 was ~40 μg protein/ml. Differences in the levels of melanocyte mitogen between rapidly proliferating versus stratifying keratinocytes were observed with and without

Table I. Mitogenic Activity toward Human Melanocytes in Keratinocyte Extracts

Medium	Additions	[³H]Thymidine incorporation
		cpm/well
PC-1	None	170
	dbcAMP	1,180
	Conditioned medium 1:1 + dbcAMP	1,900
	Conditioned medium 1:20 + dbcAMP	170
	Keratinocyte extract	3,500
	Keratinocyte extract + dbcAMP	40,300
MCDB-153	None	210
	dbcAMP	2,500
	Keratinocyte extract	1,260
	Keratinocyte extract + dbcAMP	23,900

Human melanocytes were seeded in high calcium (3.0 mM) PC-1 or low calcium (0.03 mM) MCDB-153 medium in 12-well cluster plates (80,000 cells/4-cm² well), and experimental media were added the following day. Conditioned medium was collected after 3 d incubation from keratinocyte cultures grown in MCDB-153 at ~80% confluency in which the melanocytes appeared viable. The medium was passed through a Millex-GV filter and diluted 1:1 or 1:20 with PC-1 medium. Extract was prepared from adult keratinocytes grown in MCDB-153 medium and was added at 80 μg protein/ml. The concentration of dbcAMP was 1 mM. [³H]Thymidine incorporation was carried out during the last hour of a 48-h incubation with experimental media. Values are averages of cpm from duplicate wells.

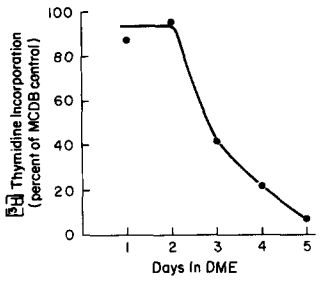

Figure 3. Kinetics of loss of melanocyte mitogenic activity during stratification of keratinocytes. Keratinocytes, derived from newborn foreskin, were propagated in MCDB-153 medium. On day 0, the cultures were split among 10 flasks (75 cm^2), half were kept in MCDB-153 medium and the other half transferred to DME. Cells were collected on each of the following 5 d, washed twice with PBS, and kept at −70°C. Cell extracts were then prepared and added at 20 μg protein/ml to duplicate wells seeded with cultures of normal human melanocytes in PC-1 medium supplemented with 1 mM dbcAMP. [^{3}H]Thymidine incorporation into melanocytes was measured during the final 3 h of a 24-h incubation with experimental media. Data points are percentages of [^{3}H]thymidine incorporation by cultures incubated with extract from keratinocytes grown in DME versus MCDB-153. The levels of [^{3}H]thymidine incorporation in the control wells fluctuated between 3,650 and 21,000 cpm, indicating that the level of mitogenic activity of keratinocytes grown in MCDB-153 is not constant.

dbcAMP. The kinetics of decrease in mitogenic activity of highly proliferating keratinocytes in response to transfer of the cultures to DME is shown in Fig. 3. There was a sharp drop in the level of mitogenic activity on the third day after transfer. This decline continued over the following days.

The mitogenic activity in keratinocytes was not limited toward melanocytes. 3T3-Swiss fibroblasts also responded to keratinocyte extract (Fig. 4). Like bFGF, the keratinocyte extract stimulated the fibroblasts independently of cAMP. As observed with melanocyte cultures, the level of the mitogen towards fibroblasts was ~10-fold higher in extracts from keratinocytes grown in MCDB-153 as compared with those grown in DME plus serum. 24 μg protein of extracts from keratinocytes grown in MCDB-153 or DME elicited mitogenic activity in the fibroblast cultures equivalent to that elicited by 5.0 and 0.5 ng/ml bFGF, respectively (Fig. 4).

MCDB-153, used to induce rapid proliferation in keratinocyte cultures, contains pituitary extract. Pituitary extract, in the presence of IBMX, is known to stimulate the proliferation of normal human melanocytes (Halaban et al., 1987) in the absence of TPA. To eliminate the possibility that the stimulatory activity in extract from keratinocytes grown in MCDB-153 was due to a pituitary factor in the medium, we deprived the keratinocytes of pituitary extract for 3 d before preparing the extract. The mitogenic activity toward melanocytes remained unaltered (data not shown).

High levels of mitogenic activity toward melanocytes were found also in extracts of dermal fibroblasts, with 5 μg protein/ml exerting optimal stimulation of growth (Fig. 5). Stimulation by fibroblast extract required dbcAMP. Conditioned medium from fibroblast cultures had no mitogenic activity toward melanocytes (data not shown).

UVB Light Induces DNA Synthesis in Melanocytes and Melanocyte Mitogen in Keratinocytes

UVB irradiation is known to increase human skin pigmentation by increasing the number of DOPA-positive melanocytes, synthesis of melanin, and the transfer of melanin to keratinocytes (Mishima and Widlan, 1967; Quevedo et al., 1969). UVB light stimulates the proliferation of melanocytes and keratinocytes in murine skin (Rosdahl and Szabo, 1978; Rosdahl, 1978). We, therefore, tested whether UVB light regulates melanocyte proliferation directly and/or indirectly by modulating the level of the melanocyte mitogen in keratinocytes. The data presented in Figs. 6 and 7 show that (*a*) UVB light stimulates DNA synthesis of human melanocytes only in the presence of TPA and (*b*) UVB light increases levels of the melanocyte mitogen in keratinocytes.

Increases in [^{3}H]thymidine incorporation by melanocytes were observed 35–50 h after the initial treatment with UVB, but only in cultures containing TPA (Fig. 6). The twofold increase in [^{3}H]thymidine incorporation in response to UVB light was followed by a 20% increase in the number of melanocytes. There were ~19,000 and 23,000 cells/cm^2 in control and irradiated dishes, respectively, at the end of the 60-h incubation. Melanocytes incubated with IBMX and cholera toxin in the absence of TPA or without additions (data not shown, but similar to those presented in Fig. 6 *C*) did not respond to UVB light. Under the latter conditions, melanocytes deteriorated rapidly, as demonstrated by the sharp decline in DNA synthetic activity. In addition to its effect on DNA synthesis, UVB light caused a 50% increase in tyrosinase activity (from 870 to 1,240 μU/mg protein) over 5 d of daily irradiation. Extracts (80 μg protein/ml) from nonirradiated and irradiated human melanocytes (collected 4 and 8 h after UVB irradiation) did not stimulate the proliferation of human melanocytes (data not shown).

The effect of UVB on the melanocyte mitogen in keratinocytes is shown in Fig. 7. At suboptimal doses of 2.5–5.0 μg protein/ml, the level of mitogenic activity in rapidly proliferating keratinocytes harvested 7 h after UVB irradiation was

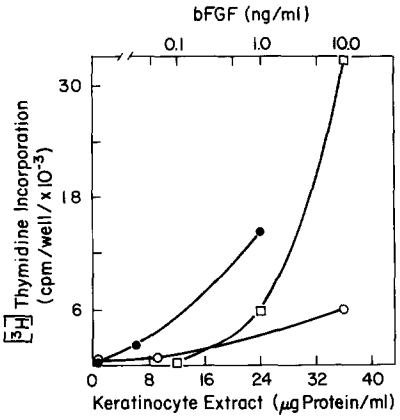

Figure 4. Dose-related mitogenic stimulation of fibroblasts by bFGF and extracts from human keratinocytes. 3T3-Swiss murine fibroblasts were seeded in 24-well cluster plates at 20,000 cells per well in DME supplemented with 10% calf serum. When cells reached subconfluent density, they were incubated in serum-free DME for 48 h. bFGF and keratinocyte extracts were added directly to each well and the assay for [^{3}H]thymidine incorporation was performed during the last 3 h of the 24-h incubation. The concentrations of bFGF are given on the top and those of extracts on the bottom scale. (□), bFGF; (●), extract from keratinocytes grown in MCDB-153; (○), extract from keratinocytes transferred to DME 9 d before. Values are averages from two wells per 3 h.

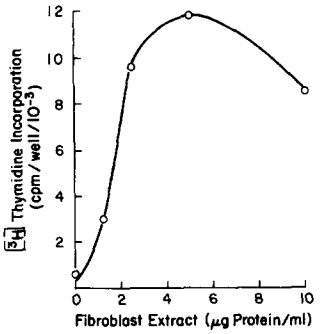

Figure 5. Mitogenic stimulation of human melanocytes by extract from human dermal fibroblasts. Fibroblasts derived from a newborn foreskin were grown in DME. Extracts from such cultures were added to melanocyte cultures in PC-1 defined medium supplemented with 1 mM dbcAMP. [^{3}H]Thymidine incorporation into melanocytes was carried out for 2 h at the end of a 24-h incubation in experimental medium. Values are averages from two wells per 1 h.

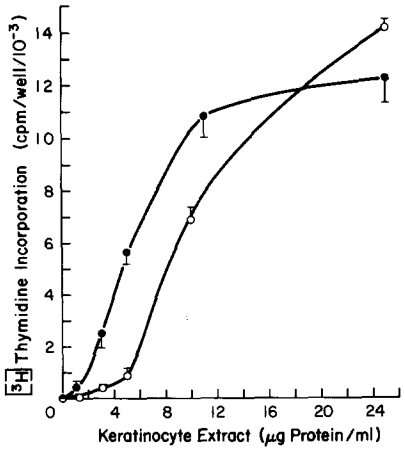

Figure 7. Increased melanocyte mitogen in UVB irradiated keratinocytes. Keratinocytes, derived from newborn foreskin, were grown in MCDB-153 medium for a month and were passaged twice. Half of the keratinocyte cultures were exposed to 150 mJ/cm^2 UVB light and harvested 7 h later (●). The control half was not exposed to UVB light (○) and was harvested at the same time as the experimental cultures. Extracts from these cultures at different concentrations were added to melanocyte cultures (24,000 cells per well) in PC-1 medium supplemented with 1 mM dbcAMP. [^{3}H]Thymidine incorporation into melanocytes was tested during the last 3 h of a 24-h incubation with experimental or control media. Values are averages from two wells per 3 h. Vertical bars indicate standard errors.

up to sixfold higher than that of nonirradiated keratinocytes. By 24 h, the mitogenic activity had returned to control levels (data not shown). A response to UVB light was observed only in keratinocytes grown in MCDB-153 but not in DME.

Interleukin 1, induced in keratinocytes in response to UVB irradiation (Kupper et al., 1987), did not stimulate the proliferation of melanocytes nor did it stimulate keratinocytes to produce higher levels of mitogenic activity toward melanocytes (data not shown).

The Melanocyte Mitogen in Keratinocytes Is bFGF

Because we had shown previously that bFGF was a natural growth factor for melanocytes, the only defined growth factor shown to be able to substitute for TPA (Halaban et al., 1987), neutralizing anti-bFGF antibodies (Halaban et al., 1987), and a synthetic peptide fragment of bFGF that inhibits bFGF activity (Shubert et al., 1987; Baird et al., 1988) as well as a bFGF-cDNA were used to probe the nature of the mitogen in keratinocytes.

As demonstrated in Table II, antibodies raised in rabbits against a synthetic peptide corresponding to a segment of the amino-terminal domain of bFGF (anti-bFGF[1-24]) and known to neutralize bFGF activity toward melanocytes (Halaban et al., 1987), neutralized at least 90% of the mitogenic activity in extracts of stratifying keratinocytes and 70% in proliferating keratinocytes. That the mitogenic activity in proliferating keratinocytes was neutralized at a lower percentage is probably due to the higher specific mitogenic activity in these cultures. With less extract (data not shown) or with more antibodies, >90% of the mitogenic activity was inhibited (Fig. 8). Fig. 8 also demonstrates that the mitogenicity of extract derived from UVB irradiated keratinocytes, supplied at 20 μg protein/ml, stimulated DNA synthesis in melanocytes to about the same level as did 160 pg/ml bFGF. Co-incubation with neutralizing anti-bFGF antibodies at dilution 1:40 reduced the mitogenicity of the keratinocyte extract by ~83% (Fig. 8) and that of 160 pg/ml bFGF by 80% (i.e., from 4,000 to 800 cpm/well per 3 h without and with anti-bFGF antibodies, respectively). The mitogenic activity in dermal fibroblasts was also completely abolished by the inhibiting anti-bFGF antibodies (data not shown).

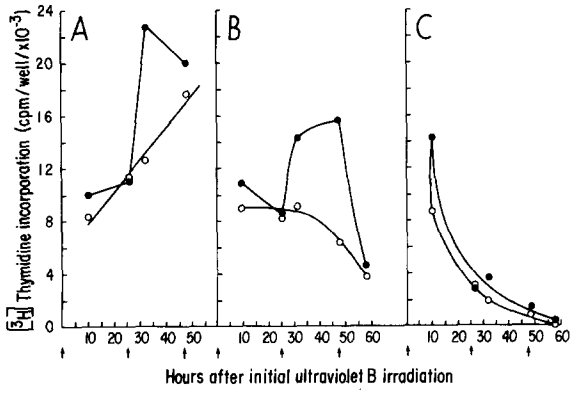

Figure 6. Incorporation of [^{3}H]thymidine by melanocytes in response to UVB light. Human melanocytes were incubated in medium containing TPA, IBMX, and cholera toxin (*A*), or TPA alone (*B*), or IBMX and cholera toxin without TPA (*C*). Duplicate cultures were exposed to 50 mJ/cm^2 UVB light (●) at various intervals as indicated by the arrows below the abscissa. Cultures not exposed to UVB light (○) served as controls. [^{3}H]thymidine incorporation was measured for 1 h, and data are averages from duplicate wells.

A synthetic peptide that blocks the binding of ^{125}I-bFGF to PC12 pheochromocytoma cells (Shubert et al., 1987), baby hamster kidney (BHK) cells, 3T3 fibroblasts and vascular endothelial cells (Baird et al., 1988), and human melanocytes (Baird, A., and R. Halaban, unpublished data) also blocked the mitogenic activity of bFGF and keratinocyte extract toward human melanocytes (Table II).

Northern blot analysis with a bovine cDNA probe for bFGF revealed that keratinocytes grown in MCDB-153 medium, but not in DME, produced bFGF gene transcripts (Fig. 9). The levels of bFGF gene transcripts varied from one culture to another (e.g., lanes *4* and *5*) in concordance with

Table II. The Melanocyte Mitogen in Keratinocytes Is Related to bFGF

Additions	[^{3}H]Thymidine incorporation
	cpm/well
A. Inhibition of mitogenic activity by anti-bFGF antibodies	
Experiment 1: keratinocytes grown in DME	
None	Not detectable
Keratinocyte extract (80 μg protein/ml)	2,100
Keratinocyte extract (80 μg protein/ml) + anti-bFGF-(1-24) serum (10 μl/ml)	150
Keratinocyte extract (80 μg protein/ml) + nonimmune serum (10 μl/ml)	2,200
Experiment 2: keratinocytes grown in MCDB-153	
None	Not detectable
Keratinocyte extract (50 μg protein/ml) + anti-bFGF-(1-24) serum (20 μl/ml)	6,000
Keratinocyte extract (50 μg protein/ml) + nonimmune serum (20 μl/ml)	19,400
B. Inhibition of mitogenic activity by a synthetic peptide fragment of bFGF	
None	Not detectable
bFGF	5,000
bFGF + (1-10)OH	5,900
bFGF + (103-146)NH_2	400
Keratinocyte extract (25 μg protein/ml) + (1-10)OH	5,200
Keratinocyte extract (25 μg protein/ml) + (103-146)NH_2	1,400
Keratinocyte extract (20 μg protein/ml) + (1-10)OH	3,700
Keratinocyte extract (20 μg protein/ml) + (103-146)NH_2	190

All additions were made to PC-1 defined medium supplemented with 1 mM dbcAMP. In experiment *1* of part *A*, extract was prepared from keratinocytes derived from adult skin grown in DME, and in experiment *2* of part *A*, and *B*, keratinocytes were derived from newborn foreskins grown in MCDB-153. Anti-bFGF-(1-24) serum was raised in rabbits as described before (Baird and Ling, 1987). bFGF (95% pure) was added at 1 ng/ml. Synthetic peptides (1-10)OH and (103-146)NH_2 were prepared as described by Shubert et al. (1987) and were added at 300 μg/ml. [^{3}H]Thymidine incorporation was carried out during the last 2-3 h of a 24-h incubation with experimental media. Data are averages from two wells.

the observed variability in the levels of the melanocyte mitogen in keratinocytes (see legend to Fig. 3). Two keratinocyte mRNA species of 7.0 and 3.7 kb hybridized to the bFGF-cDNA probe. The two cell types used as positive controls, human hepatoma (SK-HEP-1; Abraham et al., 1986*a*) and normal dermal fibroblasts (Shipley et al., 1988), contained bFGF gene transcripts of sizes identical to those found in keratinocytes (Fig. 9, lanes *6* and *7*).

Discussion

Human melanocytes differentiate not only in regard to pigment formation and cell shape but also in regard to a strict dependency on specific growth factors to be able to survive and proliferate in culture. The specific agents are bFGF (or TPA) plus substances that increase intracellular levels of cAMP. Unlike endothelial cells and fibroblasts, which are stimulated by bFGF in addition to producing this polypeptide growth factor on their own (Gospodarowicz et al., 1986; Vlodavsky et al., 1987; Schweigerer et al., 1987; Shipley et al., 1988), bFGF is undetectable in melanocytes either as gene transcript (Halaban et al., 1988) or as immunoprecipitable protein (Halaban, R., unpublished data). The absence of bFGF by these biochemical criteria was supported by biological assay. Melanocyte stimulating activity could not be detected in extracts of highly proliferative human melanocytes grown in TIP, indicating that bFGF was not induced in detectable amounts in response to mitogenic stimulation by TPA. We had shown before that human metastatic melanoma cells contained bFGF and depended on their intrinsic bFGF activity for continued proliferation, suggesting that bFGF acts as a transforming growth factor in human melanomas (Halaban et al., 1988). Our conclusion has recently been strengthened by results from another laboratory in another cell system, demonstrating that aberrant expression of

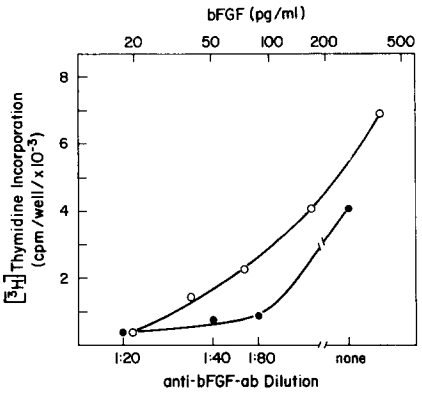

Figure 8. The mitogenic stimulation of melanocytes by bFGF and by extract from UVB irradiated keratinocytes is inhibited by anti-bFGF antibodies. Melanocytes were seeded in 24-well cluster plates at 40,000 cells per well in 0.5 ml PC-1 medium plus 1 mM dbcAMP. bFGF (○) extract from keratinocytes collected 7 h after UVB irradiation (20 μg protein/ml; ●) and anti-bFGF-(1-24) immunoglobulin fraction were added directly to each well. [^{3}H]Thymidine incorporation was measured during the final 3 h of a 24-h incubation with experimental media. Data are averages from two wells per 3 h. [^{3}H]Thymidine incorporation in cultures incubated with 400 and 160 pg/ml bFGF plus 1:40 dilution of anti-bFGF immunoglobin fraction was 2,100 and 800 cpm/well per 3 h, respectively. Incubation of keratinocyte extract with a nonimmune immunoglobulin fraction gave results similar to no addition (*none*). Scale at the top indicates bFGF concentration and at the bottom antibody dilution.

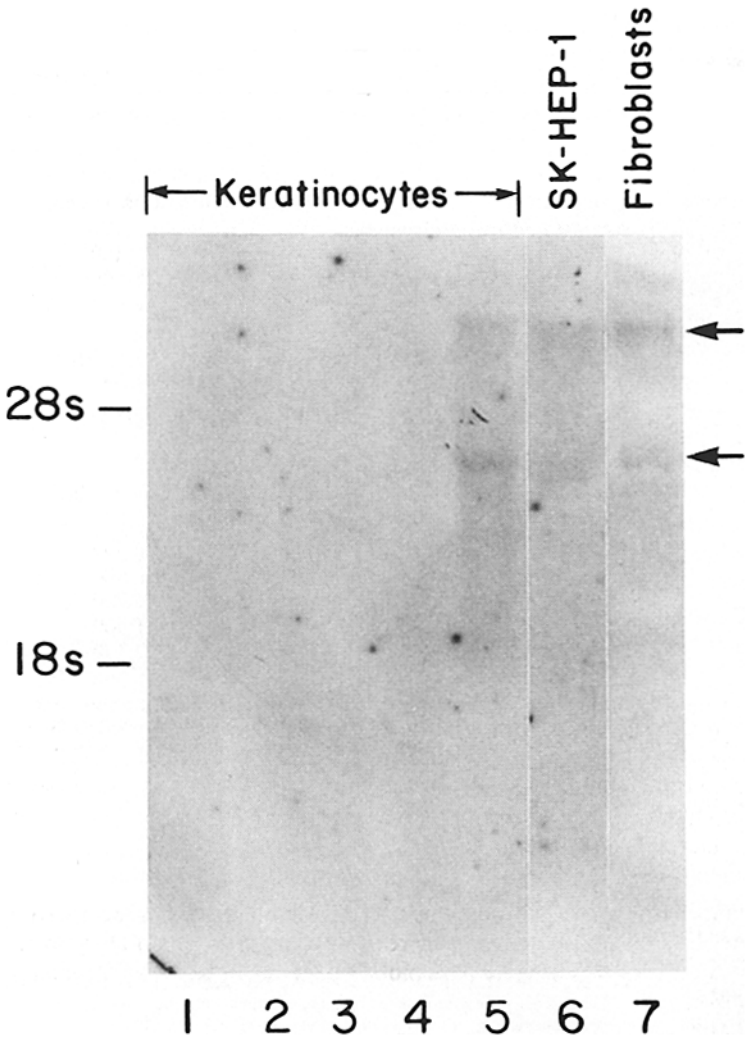

Figure 9. Expression of mRNA for bFGF in highly proliferative normal human keratinocytes. RNA samples from keratinocytes (lanes *1–5*), hepatoma SK-HEP-1 (lane *6*), and dermal fibroblasts (lane *7*) were subjected to Northern blot hybridization with a bovine cDNA probe for bFGF (a 1.4-kb Eco RI fragment of pJJ11-1). (Lanes *1* and *2*) Neonatal keratinocytes and (lane *3*) adult keratinocytes, all grown in DME; (lanes *4–5*) neonatal keratinocytes grown in MCDB-153 medium; (lanes *6* and *7*) SK-HEP-1 and fibroblasts, respectively, harvested after a 4-h stimulation with serum. RNA quantities loaded onto the gel were as follows: lanes *1* and *4–6*, 20 μg total RNA; lanes *2* and *3*, 1 μg of poly(A)$^+$ RNA; lane *7*, 10 μg total RNA. Arrows indicate 7.0- and 3.7-kb gene transcripts.

bFGF by way of transfected cDNA encoding for bFGF fused with sequences specifying a signal peptide, conferred the tumorigenic phenotype on NIH 3T3 cells (Rogelj et al., 1988).

The melanocyte mitogen in keratinocytes is probably bFGF because, as in melanoma cells, it is inhibited by two agents that inhibit the activity of bFGF. Those are antibodies to a synthetic peptide of bFGF (Halaban et al., 1987) and a synthetic fragment of bFGF that blocks the binding of bFGF to its receptor (Shubert et al., 1987; Baird et al., 1988). These two agents also inhibit the mitogenic activity of purified bFGF toward melanocytes as demonstrated here and before (Halaban et al., 1987). The presence of mRNA species that hybridize with a bFGF-cDNA probe is direct evidence that keratinocytes produce bFGF. The levels of bFGF gene transcripts in keratinocytes, like the levels of the melanocyte mitogen, are not constant. Such fluctuations may explain the failure of other investigators to detect bFGF gene transcripts in keratinocytes (Shipley et al., 1988). The two bFGF-mRNA species of 7.0 and 3.7 kb, known to be present in other tissues and cells that produce bFGF, are detected easily in keratinocytes grown in MCDB-153, the medium that promotes keratinocyte proliferation and production of the melanocyte mitogen. The bFGF-mRNA species were not detected in keratinocytes grown in DME, a medium that suppresses the levels of melanocyte mitogen in keratinocytes. Dermal fibroblasts, rich in melanocyte mitogen, also express high levels of the two gene transcripts.

Melanocytes in vivo can be triggered to divide in response to UVB (Mishima and Widlan, 1967; Quevedo et al., 1969; Rosdahl and Szabo, 1978; Rosdahl, 1978). The studies with human epidermal cells in vitro, described here, indicate that keratinocytes may regulate the proliferation of melanocytes through bFGF, whose production may be increased directly in response to UVB light or, indirectly, in consequence to UVB-induced keratinocyte proliferation. Our studies show that UVB irradiation, in addition to increasing DNA synthesis in melanocytes, raises the mitogenic activity toward melanocytes in highly proliferative keratinocytes. That UV irradiation induces DNA replication and can enhance the synthesis of selected proteins has been demonstrated for human fibroblasts (Cohen et al., 1984; Schorpp et al., 1984). These responses were suggested to have been generated through DNA damage because (*a*) other DNA damaging agents such as *N*-methyl-*N*-nitrosourea and *N*-acetoxy-2-acetylaminofluorene also induced DNA synthesis (Cohen et al., 1984), and (*b*) lower doses of UV light were sufficient to enhance the synthesis of protein in cells defective in DNA repair such as

those from patients with Cockayne's syndrome or xeroderma pigmentosum (Schorpp et al., 1984). In fibroblasts, UV light induces the expression of the same proteins that are induced by TPA (Stein et al., 1988). Similar responses to UVB or TPA appear to occur also in melanocytes. As shown here and by others, both induce DNA synthesis in melanocytes (Eisinger and Marko, 1982; Libow et al., 1988), increase synthesis of tyrosinase (Halaban et al., 1983), and increase the level of pigmentation (Friedman and Gilchrest, 1987).

UVB increases DNA synthesis in cultured melanocytes directly without intervention by keratinocytes, but only under culture conditions that are supportive of melanocyte proliferation and viability, such as in the presence of TPA. Because melanocyte proliferation depends stringently on the biochemical pathways induced by the combination of TPA (or bFGF) and elevated levels of cAMP, the stimulation of DNA synthesis by UVB irradiation in melanocytes incubated with TPA alone, but not with IBMX and cholera toxin alone, suggests that UVB light induces an increase in intracellular cAMP. An immediate effect of UVB light on the turnover of membrane phospholipids in keratinocytes has been reported recently (De Leo et al., 1984). The effect was suggested to be a direct one on membranes rather than the result of damage to nuclear DNA. Membrane perturbation in cultured amphibian melanophores by odorants results in increased cAMP and a mimicking of the hormonal action of melanotropin (Lerner et al., 1988). Our results suggest that in vivo, UVB light stimulates melanocytes indirectly through neighboring irradiated keratinocytes via increased bFGF production and directly by substituting for the cAMP requirement.

The studies described here demonstrate that in vitro, rapidly proliferating keratinocytes produce higher levels of bFGF than do stratifying keratinocytes. This is a provocative finding that is in agreement with the preferred location and/or activity of melanocytes in intact skin. Studies of [^{3}H]thymidine uptake by normal palmar epidermis of humans and monkeys have shown that 80% of the labeled nuclei are in the tips of the deep rete ridges, indicating that the keratinocytes in these areas are highly proliferative (Lavker and Sun, 1983). These deep ridges are also more heavily pigmented than shallow ridges or inter-ridge epidermis. It is thus possible that actively dividing keratinocytes stimulate neighboring melanocytes to divide and/or produce more melanin. Another well-known site of growth-associated, cyclic melanocyte activity is in anagen hair follicles, where melanocytes come to lie in close proximity to the rapidly proliferating keratinocytes that constitute the cellular bulb matrix. In mice, UVB irradiation causes an increase in the mitotic frequency in melanocytes and basal keratinocytes, and the correlation between the number of mitotic figures in basal keratinocytes and melanocytes of irradiated versus nonirradiated mouse ear is positive (Rosdahl, 1978).

The mechanism by which bFGF gets from keratinocytes to melanocytes is not clear. bFGF lacks a signal peptide (Abraham et al., 1986*a*,*b*) that would enable it to be secreted by classical exocytosis. However, in vivo, bFGF accumulates in extracellular matrices produced by vascular endothelial cells (Vlodavsky et al., 1987; Baird and Ling, 1987) and corneal epithelium and endothelium (Jeanny et al., 1987) probably through its high affinity to glycosoaminoglycans (Gospodarowicz et al., 1984). Melanocytes could be exposed to bFGF through direct contact with keratinocytes and by way of the extracellular matrix deposited by neighboring keratinocytes.

bFGF is also an angiogenic factor (Folkman and Klagsbrun, 1987), and the finding that highly proliferative keratinocytes produce bFGF may thus explain clinical conditions that involve a combination of rapid proliferation of keratinocytes and microvascular endothelial cells, such as occur in wound healing and psoriasis.

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