Stimulation of Growth of Keratinocytes by Basic Fibroblast Growth Factor

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Numerous heparin-binding growth factors active in different types of cells have recently been shown to belong to the family of fibroblast growth factors. Because these factors are active in some types of epithelial cells, we tested the activity of basic fibroblast growth factor (bFGF) from bovine brain in human keratinocyte cultures. bFGF stimulated thymidine incorporation and cellular proliferation in these cultures with half-maximal activity at approximately 60 pg/ml (4×10^{-12} M). Stimulation of thymidine incorporation was associated

with increased nuclear labeling after 22 h in the presence of bFGF under the same conditions used in the thymidine incorporation assay. bFGF was nearly as effective as epidermal growth factor (EGF) in stimulating keratinocyte growth and substantially less effective than crude placental extract, and was not additive with EGF in stimulating thymidine incorporation or proliferation of cells. The findings indicate that bFGF is a potent growth factor for keratinocytes. *J Invest Dermatol* 90: 767-769, 1988

Recent studies suggest that several independently discovered growth factors active on different types of cells are identical with or closely related to acidic or basic fibroblast growth factor (FGF) [1-3]. In some cases these heparin-binding factors have been shown to stimulate epithelial cells; Uhlrich et al. [4] described stimulation of bovine lens epithelial cells by FGF, and Crabb et al. [5] showed that a heparin-binding growth factor from brain stimulated prostatic epithelial cells. We therefore tested whether FGF was active in keratinocyte cultures using both a convenient ^3H -thymidine incorporation assay [6] and measurement of cell number. We also compared the effects of FGF and placental extract, a potent stimulator of keratinocyte growth [7].

MATERIALS AND METHODS

Cell Culture Keratinocytes were cultured from newborn foreskins according to a modification [8] of the method of Rheinwald and Green [9]. Briefly, foreskins were minced with a scalpel and incubated in trypsin (Sigma catalog number T 8253, 0.25%) and ethylenediamine tetra-acetic acid (EDTA, 0.1% w/v) in buffer (trypsin-EDTA buffer) consisting of 30 mM HEPES, 4 mM glucose, 3 mM KCl, 130 mM NaCl, 1 mM sodium phosphate, pH 7.4, and 3.3 μM phenol red [10] for 18 h at 4°C, and the epithelium was removed and dissociated epidermal cells inoculated onto a mitomycin C-treated (4 $\mu\text{g}/\text{ml}$, 2 h, 37°C) 3T3-cell feeder layer in medium consisting of 50% Ham's F-12, 50% Dulbecco's modified minimal essential medium containing 5% fetal bovine serum and supplemented with cholera toxin (10 ng/ml), hydrocortisone (0.4 $\mu\text{g}/\text{ml}$), insulin (5 $\mu\text{g}/\text{ml}$), and transferrin (5 $\mu\text{g}/\text{ml}$). EGF (10 ng/ml) was added after 3 d. After 6-9 d, subconfluent keratinocyte cultures

were washed with 0.1% (v/v) trypsin (Gibco 1:250, Grand Island, NY) in trypsin-EDTA buffer to remove 3T3 cells, and keratinocytes were suspended with 0.025% trypsin (w/v) (Sigma T 8253) with 0.01% EDTA (w/v) in trypsin-EDTA buffer according to McKeehan et al. [10] and inoculated at 1×10^5 cells per 60 mm petri dish into MCDB 153 medium [11] supplemented with EGF (5 ng/ml), insulin (5 $\mu\text{g}/\text{ml}$), hydrocortisone (0.4 $\mu\text{g}/\text{ml}$), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine pituitary extract [11] (150 $\mu\text{g}/\text{ml}$), and six amino acids (histidine, 2.4×10^{-4} M; isoleucine, 7.5×10^{-4} M; methionine, 9×10^{-5} M; phenylalanine, 9×10^{-5} M; tryptophan, 4.5×10^{-5} M; and tyrosine, 7.5×10^{-5} M), and containing 0.1 mM Ca^{++} . Cells were carried in this medium and split every 4-6 d according to McKeehan et al. [10] and used between passages 2 and 8.

Assay for Growth Using Cell Counts All assays were performed in MCDB 153 medium containing 0.1 mM ethanolamine and phosphoethanolamine, 0.4 μM hydrocortisone, insulin (5 $\mu\text{g}/\text{ml}$), 0.1 mM Ca^{++} , and six added amino acids as noted above and lacking bovine pituitary extract; this is referred to as "incomplete" medium. bFGF, EGF, or placental extract were added as described in the figure legends. Keratinocytes were plated in duplicate at 1×10^4 cells per 35 mm petri dish (1.25×10^3 cells/cm²) in 3 ml of basal medium. Cells received fresh medium daily and were counted at indicated intervals in an electronic particle counter (Coulter Counter model ZBI) after suspension with trypsin and dilution in Isoton (Coulter Electronics, Hialeah, FL). Triplicate counts were performed on each dish, and the counts were averaged. Error bars indicate standard deviations. As shown in an earlier study, cells plated under these conditions do not proliferate in the absence of added growth factors [7] but remain viable for at least 24 h.

Thymidine Incorporation Assay Unless indicated otherwise in the text and figure legends, keratinocytes were plated in 24-well trays (Corning Glass Works, Corning, NY) at 1×10^4 cells per well (5×10^3 cells/cm²) in 1 ml of MCDB 153 medium supplemented as above (incomplete medium). (Concentration of thymidine is 3 μM .) Cultures were treated with or without FGF, EGF, or placental extract in duplicate or triplicate at the time of plating and incubated for 16 h at 37°C, and cultures were incubated for another 6 h in the presence of 1 μCi of ^3H -thymidine (10 Ci/mmol, ICN, Irvine,

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Abbreviations:

bFGF: basic fibroblast growth factor

EGF: epidermal growth factor

EDTA: ethylenediamine tetra-acetic acid

CA). Twenty-two hours after being plated, cultures were washed 3 times with ice-cold phosphate buffered saline and twice with cold 5% trichloroacetic acid (TCA) and solubilized with 0.1 N NaOH containing 1% sodium dodecyl sulfate. Solubilized radioactivity was counted in a scintillation counter with Safety Solve (Research Products International, Mount Prospect, IL). Error bars indicate standard deviations in the case of triplicate or one-half the range of values in the case of duplicates.

Autoradiography Keratinocytes were inoculated into 24-well trays as described above and incubated with ^3H -thymidine ($1 \mu\text{Ci}/\text{ml}$) as in the thymidine incorporation assay. The incubation was terminated by washing the culture with phosphate buffered saline at 4°C and then fixing with two 10-min incubations in absolute methanol. The dried culture was then coated in the well with Kodak nuclear track emulsion NBT-2 (Eastman Kodak Co., Rochester, NY) diluted 1:1 (w/v) with distilled water, drained for 10 min, and stored for 3 d in a light-tight desiccator. The film was developed for 10 min in Kodak Microdol X developer, rinsed, and fixed for 3 min with Kodak Rapid Fixer. The cells were counted in the 24-well tray under phase-contrast microscopy to discriminate nuclei with and without silver grains. Five hundred cells were counted per well, and each experimental point was assessed in duplicate.

MATERIALS

Chemicals used to make MCDB 153 medium, insulin, hydrocortisone, ethanolamine, phosphoethanolamine, and purified trypsin were from Sigma, St. Louis, MO. EGF was purified to homogeneity according to Savage and Cohen [12]. Placental extract was prepared as previously described [7]. bFGF purified to homogeneity as described previously [13] was a kind gift from Bradley Olwin, Ph.D.

RESULTS

In studies of thymidine incorporation, we plated cultured keratinocytes at a density of 1×10^4 cells per well in 24-well trays in incomplete medium, which contained only insulin and hydrocortisone as growth factors. Under these conditions the cells grew poorly but were readily stimulated to grow [6] by EGF, placental extract, or bovine pituitary extract added within 24 h of plating. Under these conditions bFGF was a potent stimulator of keratinocytes and was able to stimulate the cells half-maximally at less than 0.1 ng per ml and maximally at 3 ng per ml ($1.8 \times 10^{-10} \text{ M}$) (Fig 1). Stimulation was detectable at 10–30 pg/ml (10^{-12} M) (Fig 1, inset).

In order to determine what proportion of cells stimulated by bFGF were being labeled under the conditions of the thymidine assay, we determined the percentage of labeled nuclei by autoradiography in a parallel experiment using a pulse of radioactivity during the last 6 h of the incubation. The results showed that about

Figure 1. Stimulation of thymidine incorporation in keratinocytes by bFGF. Cells were plated and incubated with or without various concentrations of bFGF and assayed as described in Materials and Methods. Inset shows results of separate experiment using lower concentrations of bFGF.

Table I. Autoradiography and ^3H -thymidine Incorporation of Keratinocytes Treated With bFGF^a

Addition	% Nuclei labeled	$\bar{x}^b$	^3H -thymidine Incorporated	$\bar{x}$
None	7.6 ± 1^c	1.0	1000 ± 0^d	1.0
FGF, 3 ng/ml	27.9 ± 0.1	3.7	3910 ± 660	3.9
FGF, 1.5 ng/ml	24.9 ± 1.6	3.3	2900 ± 40	2.9
FGF 0.1 ng/ml	18.9 ± 0.2	2.5	2090 ± 170	2.1

^a Keratinocytes were plated in 24-well trays as described in Materials and Methods and FGF was added after 6 h. ^3H -thymidine was added to each well 16 h after addition of bFGF, the incubation was continued for 6 h more, and the assays were terminated. Autoradiography and thymidine incorporation were determined in parallel wells in the same experiment. Five hundred nuclei were counted in each well, and each point is from duplicate wells.

^b Fold stimulation above control.

^c Average $\pm 1/2$ range of duplicate determinations.

^d cpm, average $\pm 1/2$ range of duplicate determinations.

28% of cells were labeled after a 22-h exposure to the mitogen. The increase in labeling of nuclei (3.7 times control) was comparable to the increase in thymidine incorporation (3.9 times control) (Table I).

In order to assess the effect of bFGF on growth of keratinocytes, we plated 1×10^4 cells in duplicate in 35-mm petri dishes in medium with the same additions used in the thymidine incorporation assay and counted the cells after 5 and 10 d. Cell counts corroborated the results of the thymidine assay, although half-maximally stimulating concentrations of bFGF were slightly higher, in the range of 0.5 ng/ml (Fig 2, inset).

We also compared the effect of bFGF with stimulation by EGF or placental extract. bFGF was slightly less effective than EGF in stimulating growth of keratinocytes in measurements of cell number (Fig 2). Neither EGF nor a combination of EGF and bFGF was as effective as crude placental extract in stimulating keratinocyte growth, and the effects of bFGF and EGF were not additive (Fig 2). The results of thymidine incorporation studies confirmed that bFGF was not additive with EGF, because stimulatory concentrations of bFGF added to cells in the presence of EGF at 1 ng/ml did not enhance incorporation (Fig 3). The striking acceleration of growth between days 5 and 10 may result from conditioning of the medium by the cells.

Figure 2. Comparison of the effects of placental extract, bFGF, or EGF on keratinocyte growth. Cells were plated in 35-mm dishes in basal medium as described in Materials and Methods and then treated with or without indicated concentrations of additions. Medium was changed daily. Cells were suspended with trypsin-EDTA solution and counted in a Coulter particle counter. Open bars, day 5; hatched bars, day 10. Inset: Line graph of data for bFGF alone. $\circ$, day 5; $\bullet$, day 10. PX, placental extract.

Figure 3. Addition of bFGF does not enhance stimulation of thymidine incorporation by EGF. Cells were plated and assayed in the presence of indicated additions as described in Materials and Methods. *Hatched bars*, bFGF only at indicated concentrations; *open bars*, indicated concentrations of bFGF plus EGF at 1 ng per ml.

DISCUSSION

FGFs are usually isolated from brain and can be resolved into acidic and basic forms of 16000–19000 daltons, depending in part on the extraction procedure, by differential elution from heparin-Sepharose columns, but similar factors have been purified from other tissues and cells including pituitary, kidney, adrenal, corpus luteum, placenta, retina, thymus, macrophages, and chondrosarcoma cells [2]. These related factors have a wide target cell specificity including fibroblasts, umbilical endothelial cells, smooth muscle cells, granulosa and adrenal cortex cells, glial cells, and prostatic and lens epithelial cells [2,4,10].

Our results show that bFGF is clearly a potent stimulant of human keratinocyte growth in culture as assessed by incorporation of ³H-thymidine (Fig 1) and by cell counting (Fig 2). It is active in the range of 10⁻¹⁰ to 10⁻¹² M in stimulating thymidine incorporation and slightly less active in stimulating proliferative growth of cells, which requires prolonged exposure of cells to the factor. Apparent reduction of activity in such experiments could be produced by binding of the very basic factor to proteins such as albumin included in the medium used to dilute the bFGF, by instability of bFGF in the medium [14], or possibly by consumption of the factor by the cells. In order to maximize the effect of added bFGF, we changed medium and added new bFGF daily in experiments measuring cell number. Thymidine incorporation, an accurate indicator of growth promotion under the conditions of our assay [6], requires shorter incubations and may be less affected by such problems.

EGF and bFGF are approximately equally effective in stimulating keratinocytes. The fact that bFGF does not increase stimulation in

the presence of EGF, even though greater stimulation is possible in the presence of the potent growth stimulator (Fig 2), crude placental extract, suggests that the two agents may be acting through similar mitogenic pathways, although not through the same receptor, because EGF and FGF do not compete for binding [13].

bFGF can act as a mitogen or can stimulate development of the differentiated phenotype in different target cells [2]. Its role in the epidermis and its interaction with other growth factors in promoting keratinocyte growth remain to be examined.

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