Effect of Basic Fibroblast Growth Factor (bFGF) and Insulin-Like Growth Factors Type I (IGF-I) and Type II (IGF-II) on Adult Human Keratinocyte Growth and Fibronectin Secretion

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Effects of growth factors on keratinocyte migration and proliferation are of interest as an indication of their potential use in acceleration of wound re-epithelialization. Various growth factors were examined for effects on normal adult human keratinocyte growth and fibronectin (Fn) secretion for cells cultured in serum-free medium. Accumulation of Fn in the medium of cells grown with H + I + EGF + BPE paralleled growth during the exponential phase and declined as the cells approached confluence. Cells maintained in low Ca^{++} (0.15 mM) post-confluence and fed daily to prevent cornification continued to accumulate Fn in the medium, while those grown continuously in 1.2 mM Ca^{++} ceased Fn secretion at confluence. EGF, bFGF, and transforming growth factor-β (TGFβ) stimulated keratinocyte Fn secretion in correlation with literature reports on the ability of these factors to stimulate the migration of these cells. In contrast, despite its marked effects on cell growth, BPE was found to consistently reduce the amount of Fn found in the medium when added to cultures containing either EGF or bFGF. Addition of BPE to cultures containing EGF or bFGF stimulated growth to the same extent, indicating that the effects of BPE on keratinocyte growth are not solely due to its content of bFGF. *J Invest Dermatol 94:777–780, 1990*

Recently there has been an increasing interest in the use of cell releasates and recombinant purified growth factors for acceleration of cutaneous wound healing [1–5]. A number of clinical studies have now shown complete healing of chronic leg ulcers using grafted keratinocytes previously maintained in culture [6–8], suggesting that re-epithelialization of such wounds may be the most important first step in normal skin restoration. Such studies imply the possibility that the grafted cells are secreting growth factors which stimulate the patient's own recalcitrant keratinocytes to initiate migration across the wound bed [6,9]. Since Fn has been shown to be important for keratinocyte migration and growth both in vitro [10] and in vivo [11,12], this work has explored the effects of certain growth factors on overall growth of normal adult human keratinocytes as well as on Fn secretion into the medium.

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Abbreviations:
bFGF: basic fibroblast growth factor
BPE: bovine pituitary extract
EGF: epidermal growth factor
ELISA: enzyme-linked immunoabsorbent assay
Fn: fibronectin
H: hydrocortisone
I: insulin
IGF-I: insulin-like growth factor type I
IGF-II: insulin-like growth factor type II
IgG: immunoglobulin G
KBM: Clonetics modified MCDB153 basal medium
KGM: Clonectics KBM supplemented with I + H + EGF
PMA: phorbol 12-myristate 13-acetate
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
TGFβ: transforming growth factor beta

MATERIALS AND METHODS

Normal adult human keratinocytes were purchased from Clonetics, San Diego, CA, and were grown in modified MCDB153 obtained from Clonetics supplemented with various growth factor combinations. Cells were obtained as proliferating secondary cultures and were seeded into tertiary culture for experiments at 25,000 cells per 35 mm well when about 80% confluent. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), hydrocortisone (H), insulin (I), and bovine pituitary extract (BPE) were present at 10 ng/ml, 10 ng/ml, 0.5 μg/ml, 5 μg/ml, and 70 μg/ml, respectively. EGF, I, H, and BPE were obtained from Clonetics. bFGF (from Amgen, Thousand Oaks, CA) was used for the data in Table II, and bFGF (from Collaborative Research, Bedford, MA) was used for the data in Fig 3. IGF-I and IGF-II were produced at Eli Lilly [13,14]. TGFβ was a gift from Dr. Charles Frolik, Eli Lilly [15]. Growth was determined by counting cells released by trypsin-EDTA in a Coulter counter after dilution in Isoton. Growth media were changed 24 h after plating and at 2-d time intervals thereafter or as indicated in tables and figures. Cornified cells were identified by staining with rhodanile blue according to Rheinwald and Green [16].

Medium Fn levels were determined using an indirect inhibition ELISA [17] on samples added to microtitration plates the same day the media were harvested. Wells were coated with 2000 ng of human plasma Fn (Collaborative Research, Bedford, MA). The anti-human Fn antiserum used at a dilution of 1/2000 was also from Collaborative Research. Antibody bound to microtiter plates was determined using alkaline phosphatase-labeled protein A from

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Boehringer Mannheim, Indianapolis, Ind. Plates were read in a Vmax plate reader from Molecular Devices, Menlo Park, CA. Electrophoresis was carried out in 1.5 mM, 7.5% polyacrylamide gels according to Laemmli [18]. Gels were transblotted to nitrocellulose for 18 h at 4°C in a semidry electroblotter. Total protein on blots was stained with colloidal gold from BioRad, Richmond, CA. Immunoblots were incubated with anti-human Fn followed by horseradish peroxidase-labeled goat anti-rabbit IgG (BioRad) diluted 1/3000 and 4-chloro-1-napthol (BioRad). Collagen types I and III, laminin, and PMA were from Sigma, St. Louis, MO. Collagen type IV was from Collaborative Research. Statistical evaluation of data was by Student t test.

RESULTS

Binding of the commercial antiserum used to microtiter plates was inhibited 50% in the ELISA by human plasma Fn at 50 ng/ml while greater than 10,000 ng/ml of the other connective tissue proteins tested (collagen Types I, III, and IV and laminin) were required to affect antibody binding. Figure 1 confirms by Western blot analysis the specificity of the antiserum used which reacted with human Fn but not with collagen types I or III or with laminin. Figure 2 compares Fn secretion versus cell growth for cells grown in KGM + BPE in low Ca^{++} (0.15 mM) versus high Ca^{++} (1.2 mM). The medium was changed daily after day 5 to prevent the cells in low Ca^{++} from differentiating [19]. Although cells in both groups continued to grow for the duration of the experiment, the cell number in high Ca^{++} was greater from day 5 forward, presumably due to increased degree of stratification [19]. While the cells in low Ca^{++} continued to secrete Fn into the medium post-confluence, the high-Ca^{++} group ceased Fn production after day 5. Cornification in the high-Ca^{++} group was verified by rhodanile blue staining.

Figure 3 shows the effect on cell growth of adding EGF or bFGF to basal medium with H + I. Growth was significantly increased to the same extent ($p < 0.05$) by both growth factors, and combining them did not further enhance growth. Addition of IGF-I to cells grown in the presence of EGF and H stimulated growth sevenfold at 100–175 ng/ml (data not shown). Table I shows a dose response for IGF-II added in place of insulin to basal medium containing H + EGF. With regard to cell number, IGF-II had an optimal effect at 100 ng/ml in this experiment, equivalent to 5 μg/ml insulin, and

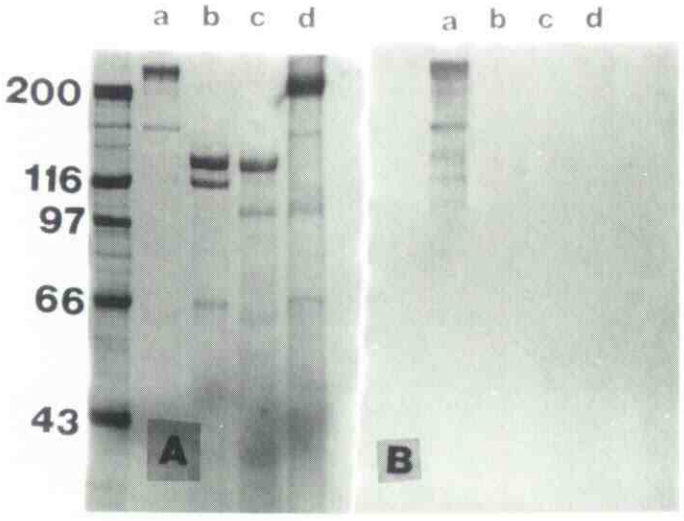

Figure 1. Immunoblot analysis of anti-Fn antiserum. Proteins were separated by SDS-PAGE and were transblotted to nitrocellulose. Equal amounts of each sample were applied to the left and right half of the gel, and after transblotting the blot was cut in half. The left half *(A)* was stained with colloidal gold, while the right half *(B)* was immunostained with the antiserum. Lanes were: *a)* 5 μg human Fn, *b)* 10 μg collagen type I, *c)* 10 μg collagen type III, and *d)* 10 μg laminin. Only reactivity with Fn was observed.

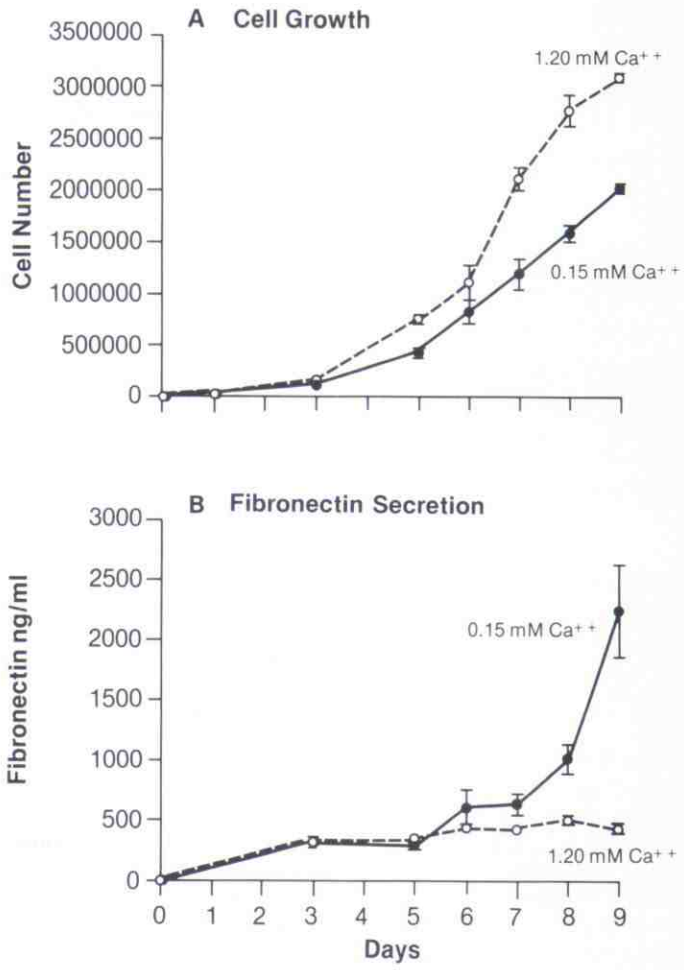

Figure 2. Effect of continuous exposure to low versus high Ca^{++}. Cells were grown in KGM + BPE in the presence of the indicated concentrations of Ca^{++}. *A)* Cell growth. *B)* Fn secretion. Cells were fed daily after day 5.

appeared to be somewhat less effective at higher doses. Accumulation of Fn in the medium in this experiment, determined at 2-d time intervals, in general was highest on a per cell basis under conditions where growth was maximal (Table I).

Table II compares the effects of EGF and bFGF on cell growth and medium Fn accumulation in the presence of H + I and BPE. Addition of EGF or bFGF to medium containing both H + I provided only a slight increase in cell number in this experiment. Addi-

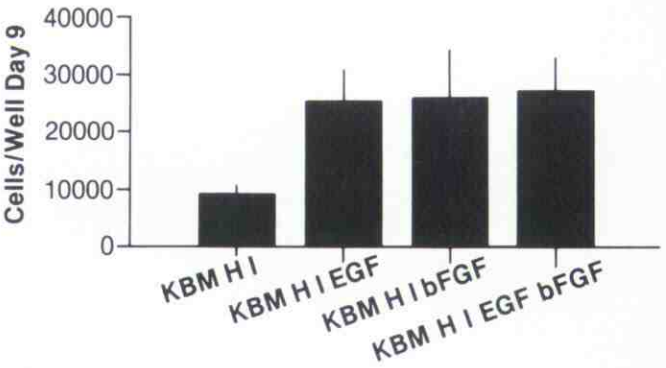

Figure 3. Effect of EGF versus bFGF on keratinocyte growth. Cells were plated out in basal medium with H and insulin I and were switched to the growth media indicated. Cell counts were determined on day 9. Data are the mean ± SD from three wells.

Table I. Dose Response effect of IGF-II on Keratinocyte Growth and Fibronectin Secretion

Growth Medium	Cells/Well Day 10[a]	Cumulative Fn[b] (ng/ml 10^6 cells)
KBM H EGF	94, 100 ± 6,000	3,500
KBM H EGF I	310,260 ± 45,000 (329)	6,600 (188)
KBM H EGF IGF-II[c]		
10	168,130 ± 24,000 (179)	1,500 (43)
100	321,870 ± 21,700 (342)	4,700 (134)
250	212,900 ± 61,000 (226)	3,200 (91)
500	272,270 ± 47,580 (289)	4,000 (114)
1000	191,000 ± 52,000 (203)	1,300 (37)

[a] Cells were plated out in KGM with BPE. Cell count at day 1 was 30,267 ± 3,700. Data are the mean ± SD from three wells.
[b] The total amount of Fn accumulated in the medium by day 10 as determined by ELISA at 2 time intervals. Data are on media pooled from three wells for each condition.
[c] Number are ng/ml. Numbers in parentheses are percent of control (KBM H EGF).

tion of BPE to basal medium with H + I + EGF or bFGF caused an additional threefold increase in cell number in both cases. Both EGF and bFGF increased Fn accumulation when added to medium with H and I. Although addition of BPE to media with either H + I + EGF or bFGF clearly increased cell number, the amount of Fn accumulated in the medium between days 5 and 7 was strikingly reduced.

Table III shows an experiment designed to evaluate the effects of various growth factors on Fn accumulation in the medium over a 24-h time interval. Cells were all grown in KGM without BPE for a period of time and were then switched to KBM for 2 d to remove the KGM growth factor effects. After this period without growth factors, they were cultured in the presence of the various combinations shown for 48 h and Fn was determined on media collected during the last 24 h. Addition of TGFβ or PMA to KGM + BPE inhibited cell growth as expected. Addition of EGF to basal medium + H + I caused a significant increase in Fn accumulation ($p < 0.01$). As seen in Table II, addition of BPE to KGM clearly reduced the amount of Fn found in the medium ($p < 0.01$). TGFβ was able to overcome this effect of BPE and stimulate Fn accumulation ($p < 0.01$). Addition of PMA to KGM + BPE had no significant effect on Fn secretion.

DISCUSSION

The addition of various growth factor combinations to adult human keratinocytes grown in serum-free medium was investigated in this study for effects on overall cell growth as determined by cell counting and for effects on Fn secretion into the medium as determined by ELISA. Insulin-like growth factors type I and type II were able to substitute for insulin and stimulate cell growth. bFGF had effects on growth approximately equivalent to EGF. Although Fn accumulation in the medium often was correlated with growth stimulation (Fig 2, Tables I–III) notable exceptions were found in the presence of high Ca^{++} (Fig 2), BPE (Tables II and III), and TGFβ (Table III).

Both IGF-I and IGF-II stimulated cell growth to the same extent as insulin (5 μg/ml) but at far lower concentrations (100–200 ng/ml). These results are similar to recent literature reports [20,21]. The comparable effects of bFGF at 10 ng/ml to 10 ng/ml EGF (Fig 3, Table II) are also similar to recent reports for both mouse and human keratinocytes [22–24].

Accumulation of Fn in the medium of adult human keratinocytes parallels growth during the exponential phase and declines as the cells become confluent and experience growth-arrest (Fig 2). However, cells maintained in low Ca^{++} and fed daily to prevent terminal differentiation [25] continue to secrete Fn into the medium, while those grown continuously in high Ca^{++} cornify and cease Fn production (Fig 2). Both EGF and bFGF stimulate Fn accumulation, as does TGFβ (Tables II and III). EGF and TGFβ have previously been shown to stimulate Fn production by cultured keratinocytes [26,27]. In contrast, despite its marked effects on keratinocyte

Table II. Effects of EGF and bFGF on Cell Growth and Fn Secretion

Growth Medium	Cells/Well Day 7[a]	Fn Day 7[b] ng/ml	Fn Day 7[b] ng/ml/10^6 cells
KBM H I	105,867 ± 11,595	831 ± 92	7,849
KBM H I EGF	134,933 ± 6,278 (127)	2,534 ± 461	18,780 (239)
KBM H I bFGF	134,133 ± 10,825 (127)	1,673 ± 227	12,473 (159)
KBM H I EGF BPE	472,800 ± 43,863 (446)	535 ± 56	1,131 (14)
KBM H I bFGF BPE	430,200 ± 1,800 (406)	609 ± 9	1,415 (18)

[a] Cells were plated out in KGM without BPE. Cell count on day 1 was 13,600 ± 1,216. Cell counts are the mean ± SD from three wells.
[b] Fn levels per ml are the mean ± SD determined by ELISA on media removed from three wells per group representing accumulation between days 5 and 7. Numbers in parentheses are percent of control (KBM H I).

Table III. Effects of Growth Factors on 24-hour Fn Secretion

Growth Medium	Cells/Well[a]	Fibronectin[b] ng/ml	Fibronectin[b] ng/ml/10^6 cells
KBM H	384,133 ± 27,624	518 ± 87	1,348
KBM H I	441,333 ± 28,630 (115)[e]	468 ± 87	1,060 (79)
KBM H I EGF(KGM)	556,867 ± 66,866 (145)	2,662 ± 669	4,780 (354)
KGM BPE	705,733 ± 30,264 (184)	745 ± 41	1,056 (78)
KGM BPE TGFβ[c]	405,600 ± 12,179 (105)	2,982 ± 1129	6,618 (491)
KGM BPE PMA[d]	383,933 ± 7798 (100)	553 ± 18	1,440 (107)

[a] Cells were grown in KGM without BPE until visually half confluent (day 8). They were switched to KBM for 2d with medium change after 24h. At 48h after the KBM switch, they were changed to the test media indicated. Media were changed to fresh test media after 24h, and after an additional 24h cells were counted and media were removed for Fn analysis. Cell counts are the mean ± SD from three wells per group.
[b] Fn levels as determined by ELISA are the mean ± SD from three wells.
[c] TGFβ was present at 5 ng/ml.
[d] PMA was present at 100 nM.
[e] Numbers in parentheses are percent of control (KBM H).

growth, BPE was consistently found to decrease the amount of Fn detected when added to media with either EGF or bFGF (Tables II and III). This effect of BPE may partially explain its inability to support clonal growth of keratinocytes without addition of other growth factors [25]. The equivalent effects of BPE on keratinocytes grown in medium with EGF or bFGF indicates that its growth stimulatory properties are not solely due to bFGF. Fn production by keratinocytes may play a role in wound re-epithelialization [28]. Stimulation of Fn production by EGF, bFGF, and TGFβ correlates with the demonstrated enhancement of keratinocyte migration by these agents [26,29,30]. Clearly, Fn production does not always correlate with overall keratinocyte growth because TGFβ (enhanced Fn) is an inhibitor of the growth of these cells [31] while BPE (decreased Fn) is a potent growth stimulator [25]. PMA, a potent inhibitor of keratinocyte growth due to induction of terminal differentiation [32], had no effect on Fn production in this study (Table III). Because only soluble Fn found in the medium was determined in this study we cannot rule out possible growth factor effects on Fn turnover or deposition into the extracellular matrix.

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