Insulin-Like Growth Factor-I and Epidermal Growth Factor Regulate Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3) in the Human Keratinocyte Cell Line HaCaT

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The human keratinocyte cell line HaCaT has a basal phenotype and secretes an insulin-like growth factor (IGF) binding protein, IGFBP-3, which modulates its IGF-I response. Keratinocytes are highly responsive to mitogenic stimulation by IGF-I and epidermal growth factor (EGF), but the effect of these growth factors on IGFBP secretion by keratinocytes is not known. We investigated the effects of IGF-I and EGF, as well as three other skin-growth regulators, retinoic acid, basic fibroblast growth factor, and dexamethasone, on mitogenic stimulation and IGFBP-3 production in HaCaT cells. IGF-I and EGF were strongly mitogenic, whereas retinoic acid, basic fibroblast growth factor, and dexamethasone were not significantly mitogenic. IGF-I increased the level of IGFBP-3 in cell-conditioned medium by up to twofold, whereas EGF caused a twentyfold reduction in IGFBP-3. Retinoic acid and basic fibroblast growth factor had only minor effects on IGFBP-3 and dexamethasone had no effect. IGF-I stimulation of IGFBP-3 did not involve increases in IGFBP-3 mRNA; however, EGF, consistent with its effect on IGFBP-3 protein, caused a fivefold reduction in IGFBP-3 mRNA. In summary, EGF profoundly inhibited IGFBP-3 synthesis in basal keratinocytes, whereas IGF-I increased IGFBP-3 levels by a post-transcriptional mechanism. We hypothesize that by inhibiting IGFBP-3 production in basal keratinocytes, epidermal mitogens such as EGF might stimulate epidermal growth indirectly by increasing local IGF-I availability. ***Key words: skin/epidermis. J Invest Dermatol 105:602–607, 1995***

Insulin-like growth factor I (IGF-I), which is produced in the dermis, is a potent mitogen for keratinocytes [1–3]. The essential role of IGF-I in normal epidermal growth was recently illustrated in transgenic mice that lacked the IGF-I receptor: the mice showed a severe inhibition of cell progression from the basal to the spinous layer of the epidermis, resulting in thin, translucent skin [4]. Because it is the basal cells of the epidermis that have the highest concentration of IGF-I receptors [5], it is likely that the basal layer is an important cellular target for IGF-I. We have previously demonstrated that the cell line HaCaT, which has the characteristics of a purely basal epidermal keratinocyte [6], synthesizes predominantly IGF binding protein-3 (IGFBP-3) in culture [7]. *In situ* hybridization studies from our laboratory show that IGFBP-3 synthesis in skin is indeed concentrated in the basal layer [8]. The HaCaT cell line thus provides a valuable model for studying IGFBP-3 regulation in the basal epidermis. Because IGFBPs [9] and IGFBP-3 in particular [7,10,11] have been shown to significantly influence the response to IGF-I in a variety of systems, IGFBP production by basal keratinocytes certainly has the potential to affect their response to IGF-I *in vivo*. It follows that other skin-growth regulators could alter the IGF-I responsiveness of basal keratinocytes by regulating IGFBP-3.

We therefore investigated the effect of several hormones known to influence skin growth, including IGF-I, on IGFBP production by basal keratinocytes. Like IGF-I, epidermal growth factor (EGF) is a potent mitogen for keratinocytes [12] and its target in the epidermis, the EGF receptor, is also largely limited to the basal keratinocytes [13,14]. Basic fibroblast growth factor (bFGF) is another mitogen of cultured keratinocytes [1,15] and is widely used to stimulate keratinocyte growth in serum-free culture [16], but its effects on IGFBP-3 production are not known. Retinoic acid and corticosteroids are widely used in the treatment of psoriasis and other skin disorders. Retinoic acid is mitogenic in cultured skin keratinocytes [17] and inhibits the differentiation of keratinocytes [18] including HaCaT cells [19]. Although both retinoic acid [20] and dexamethasone [21] alter IGFBP expression in other cell types, their effects on IGFBP production in the epidermis have not been investigated.

The findings presented here indicate that IGF-I and EGF do alter IGFBP-3 production by HaCaT basal keratinocytes, while the other hormones tested had little effect. We examine these observations in detail and discuss their relevance in normal and disordered epidermal growth.

MATERIALS AND METHODS

Materials Recombinant human IGF-I was a gift from Dr. A. Skottner (KabiPharmacia, Peptide Hormones, Sweden). Recombinant human EGF [3-^{125}I-iodotyrosyl]IGF-I (2000 Ci/mmol) and [methyl-^{3}H]thymidine (6.70

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Abbreviation: IGFBP, insulin-like growth factor binding protein.

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Ci/mmol) were from Amersham, Sydney, Australia. Bovine bFGF and random primed DNA labeling kit were from Boehringer Mannheim (North Ryde, NSW, Australia). Dexamethasone was from Sigma Chemical Co, St. Louis, MO, and trans-retinoic acid was provided by Dr. U. Novak (Royal Melbourne Hospital, Melbourne, Australia). The cDNA for human IGFBP-3 [22] was kindly provided by Dr. S. Shimasaki (Whittier Institute, La Jolla, CA) and the synthetic oligonucleotide complementary to 18S ribosomal RNA was from Dr. A. Herington, Royal Children's Hospital, Melbourne, Australia.

Cells The differentiated human keratinocyte cell line, HaCaT [6], was kindly provided by Prof. N. Fusenig, German Cancer Research Centre, Heidelberg, Germany. Cells at passage numbers 33 to 36 were maintained as monolayer cultures in 5% CO_2 at 37°C in keratinocyte serum-free medium (Gibco), containing EGF and bovine pituitary extract as supplied. Media containing fetal bovine serum were avoided because of the high content of IGF-I binding proteins in serum.

Assay of Mitogenic Activity The mitogenic effect of each hormone on HaCaT cells was assayed using thymidine incorporation and direct cell counting.

For thymidine incorporation assays, cells were grown to 3 d post confluence in 2-cm^2 wells with daily medium changes of keratinocyte serum-free medium, and the medium was changed to Dulbecco's modified Eagle's medium (DMEM) (Cytosystems, Australia), with the following additions: 25 mM HEPES, 0.19% (w/v) sodium bicarbonate, 0.03% (w/v) glutamine (Sigma), 50 IU/ml penicillin, and 50 μg/ml streptomycin (Flow Laboratories). After 24 h, hormones were added to triplicate wells at the concentrations indicated in 0.5 ml fresh DMEM containing 0.02% bovine serum albumin (Sigma molecular biology grade) and incubated for a further 21 h before measuring thymidine incorporation rates. [^{3}H]-thymidine (0.1 μCi/well) was added and the cells incubated for a further 3 h. The medium was then aspirated and the cells washed once with ice-cold phosphate-buffered saline and twice with ice-cold 10% trichloroacetic acid. The trichloroacetic acid–precipitated monolayers were then solubilized with 0.25 M NaOH (200 μl/well) and transferred to scintillation vials, and radioactivity was determined by liquid scintillation counting (Pharmacia Wallac 1410 liquid scintillation counter). Percent thymidine incorporation compared to untreated cells was expressed as the mean of triplicates ± SD.

To measure effects on cell number, we used the cell-counting assay of Schulz *et al* [23] developed specifically for the HaCaT cell line. Briefly, HaCaT cells were plated at 5000 cells per well (approximately 10% confluent) in a 96-well tissue-culture plate (Falcon number 3072, Becton Dickinson Labware) in keratinocyte serum-free medium. After 24 h, medium was changed to DMEM, and after a further 24 h hormones were added in quadruplicate wells in 200 μl DMEM. After 4 d, cells were fixed and counted exactly as described [23].

Western Ligand Blotting For analysis of IGFBPs by Western ligand blot, cells were grown to 3 d post confluence in 2-cm^2 wells with daily medium changes of keratinocyte serum-free medium, and the medium was changed to DMEM as above. After 24 h, hormones were added in fresh DMEM daily for 4 d. After the final addition, the cells were incubated for 2 d, and the cell-conditioned medium was collected. The cells were trypsinized and counted in a Coulter Industrial D Counter, Coulter Bedfordshire, UK.

HaCaT conditioned medium (250 μl) was concentrated by adding 750 μl cold ethanol, incubating at −20°C for 2 h and centrifuging at 16,000 × g for 20 min at 4°C. The resulting pellet was air dried, resuspended thoroughly in non-reducing Laemmli sample buffer, heated to 90°C for 5 min, and separated on 12% sodium dodecylsulfate–polyacrylamide gel electrophoresis according to the method of Laemmli [24]. Separated proteins were electrophoretically transferred to nitrocellulose membrane (0.45 μm, Schleicher and Schuell, Dassel, Germany) in a buffer containing 25 mM tris, 192 mM glycine, and 20% (v/v) methanol. IGFBPs were then visualized by the procedure of Hossenlopp *et al* [25], using [^{125}I]–IGF-I, followed by autoradiography. Autoradiographs were scanned in a BioRad Model GS-670 Imaging Densitometer and band densities were determined using the Molecular Analyst program.

Autoradiographs were exposed so that band optical densities were directly proportional to relative amounts of IGFBP-3 (empirically determined, data not shown). Relative band intensities were then standardized against the number of cells in the assayed well. Cell numbers varied by less than 20%. The data shown represent relative IGFBP-3 levels compared to control untreated cells and are the mean relative level from three separate experiments ± SD.

Northern Analysis For analysis of IGFBP mRNAs, HaCaT cells were grown to 3 d post-confluence in 60-cm^2 dishes with daily medium changes of keratinocyte serum-free medium, and the medium was changed to DMEM as above. After 24 h, hormones were added in fresh DMEM daily for 2 d. Twenty-four hours after the second addition, total RNA was prepared by the method of Chomczynski and Sacchi [26] and analyzed by Northern blotting as described below.

Total RNA (20 μg) was denatured at 55°C for 1 h in 1 M glyoxal, 50% dimethylsulfoxide, 10 mM $NaPO_4$, pH 7.0, and electrophoresed in a 1% agarose gel in 10 mM $NaPO_4$, pH 7.0. After transfer to Hybond-N (Amersham) in 20 × SSC (3 M NaCl, 0.3 M trisodium citrate dihydrate, pH 7.0), RNA was ultraviolet cross-linked (2.5 J/cm^2) and the membrane pre-hybridized for 5 h at 48°C and probed overnight at 48°C in a solution containing 2.5 × SSC (0.38 M NaCl, 0.038 M trisodium citrate dihydrate, pH 7.0), 50% (v/v) formamide, 0.05% (w/v) sodium pyrophosphate, 5 × Denhardt's solution, 25 mM sodium phosphate, 0.5% (w/v) sodium dodecylsulfate and 100 μg/ml of boiled herring sperm DNA. The membrane was probed with cDNAs for human IGFBP-3 or -4 labeled with α-[^{32}P]-dCTP (Amersham, 3000 Ci/mmol) by random oligonucleotide priming. The membrane was then washed for 3 × 10 min in 2 × SSC/0.1% sodium dodecylsulfate at 48°C and autoradiographed overnight. To correct for possible differences in RNA loading, the membrane was re-probed with a synthetic oligonucleotide complementary to 18S ribosomal RNA after stripping of hybridized, labeled cDNAs in several changes of 5 mM tris HCl, pH 8.0, 2 mM ethylenediaminetetraacetic acid, 0.1 × Denhardt's, at 65°C for 2 h. The membrane was pre-hybridized for 5 h at 65°C in the same pre-hybridizing solution as before except that formamide was omitted. The 18S oligonucleotide was end-labeled with γ-[^{32}P]-ATP (3000 Ci/mmol) with T4 polynucleotide kinase (Boehringer Mannheim) and added to the prehybridizing solution. The membrane was probed overnight at 65°C and washed for 2 × 10 min in 2 × SSC/0.1% sodium dodecylsulfate at 65°C, then for 4 × 20 min in 0.1 × SSC/0.1% sodium dodecylsulfate at 65°C, and autoradiographed. Autoradiographs were scanned and band intensities determined as above. Relative band intensities were then standardized against the density of the 18S ribosomal RNA bands.

Statistical Analysis The statistical significance of differences between mean values was determined using Student *t* tests.

RESULTS

IGF-I and EGF Are Mitogenic in HaCaT Cells The mitogenic effects of the hormones on HaCaT cells were examined by measuring both thymidine incorporation rates and cell-growth rates in response to hormone treatment. For thymidine incorporation assays, 1-d post-confluent monolayers of HaCaT cells were incubated for 24 h in growth factor–free DMEM, hormones were added, and thymidine incorporation rates were then measured after 21 h as described in *Materials and Methods*. The rate of thymidine incorporation has been shown to be a valid indicator of mitogenic activity in keratinocytes [12]. Cell-growth rates were measured by incubating HaCaT cells, initially at 10% confluence, with each hormone and determining the relative number of cells per well after 4 d as described in *Materials and Methods*.

As shown in **Fig 1 (*a–f*)**, IGF-I and EGF increased thymidine incorporation rates by 8.5- and fivefold and cell number by 1.35- and 1.7-fold, respectively. bFGF (30 ng/ml) caused a twofold increase in thymidine incorporation rate without affecting cell-growth rate. The other hormones, dexamethasone and retinoic acid, were not mitogenic by either assay, retinoic acid actually inhibiting cell growth at 10^{-6} M **(Fig 1 *a,d*)**.

EGF Inhibits IGFBP-3 Production by HaCaT Cells Whereas IGF-I Increases IGFBP-3 Levels The five hormones were tested for their ability to regulate IGFBP-3 production by HaCaT cells. After incubation of the cells in growth factor–free DMEM for 24 h, hormones were added at the same concentrations used in the mitogenic assays, and the cells were then grown for 4 d with daily changes of the hormones in fresh medium. This repeated treatment was found to be necessary to observe significant changes in IGFBP-3 levels in conditioned media. **Figure 2*a*** shows analysis by Western ligand blotting of the amount of IGFBP-3 appearing in the 48-h period following the final addition of hormones at maximal concentrations. Most obvious is the marked inhibition of IGFBP-3 by EGF. IGF-I caused an increase in IGFBP-3 band intensity, whereas the other hormones had little detectable effect. Band densities were quantified by optical scanning as described in

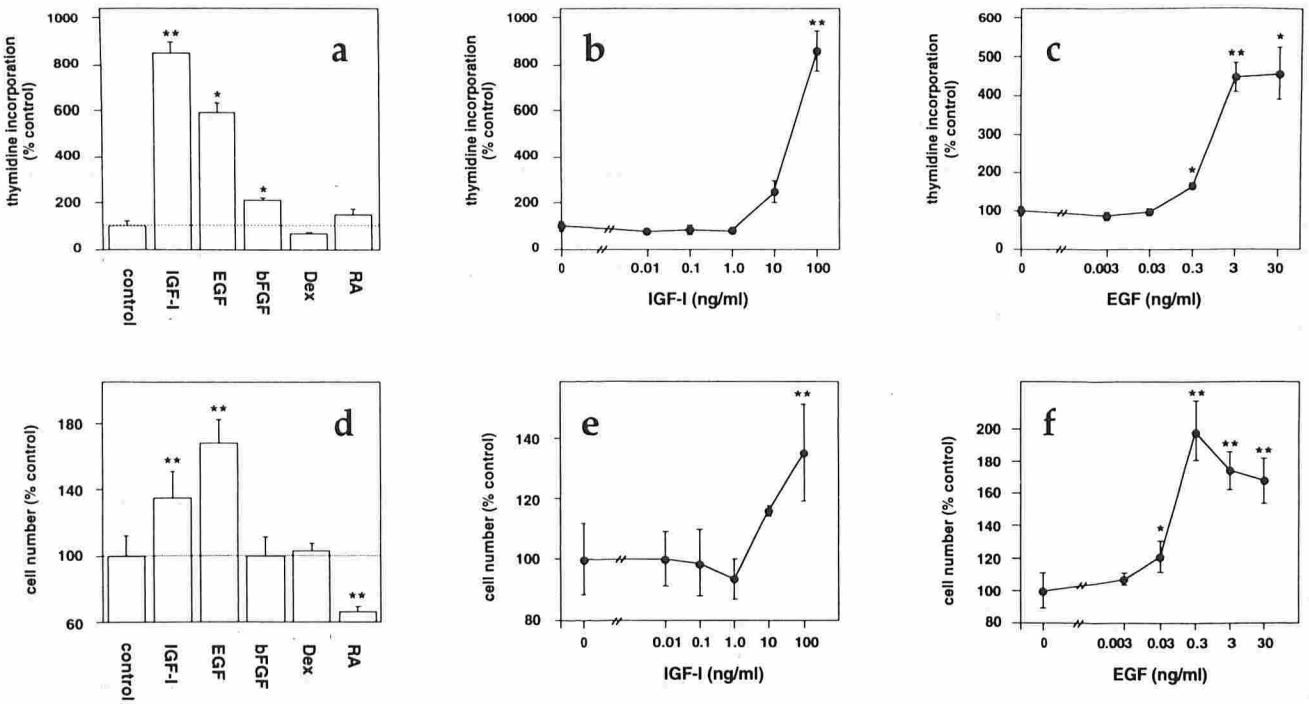

Figure 1. IGF-I and EGF are mitogenic in HaCaT cells. Effect of hormone treatment on thymidine incorporation rates (*a–c*) and cell growth rates (*d–e*). After 24 h in growth factor–free medium, post-confluent monolayers of HaCaT cells were incubated with each hormone at the concentrations indicated. After 21 h, thymidine incorporation rates were measured as in *Materials and Methods (a–c)*. For cell-growth assays, HaCaT cells were plated at 10% confluence and incubated with each hormone for 4 d. Then relative cell number was determined as in *Materials and Methods* (*d–e*). *Control*, untreated; *IGF-I*, 100 ng/ml IGF-I; *EGF*, 30 ng/ml EGF; *bFGF*, 30 ng/ml bFGF; *Dex*, 10^{-7} M dexamethasone; *RA*, 10^{-6} M retinoic acid. Data points represent means of triplicate (thymidine incorporation) or quadruplicate (cell counting) wells ± SD. $^{*}p < 0.01$, $^{**}p < 0.001$ versus control group.

Materials and Methods. As shown in **Fig 2*b***, IGF-I caused a variable (mean, twofold) increase in IGFBP-3 in the medium, whereas EGF caused a twentyfold reduction in IGFBP-3 levels. Retinoic acid and bFGF slightly reduced IGFBP-3 levels, whereas dexamethasone had no significant effect.

The concentration dependence of these effects is shown in **Fig 3*a–e***. Stimulation of IGFBP-3 levels in conditioned medium by IGF-I was half-maximal at approximately 10 ng/ml **(Fig 3*a*)**. The reduction in IGFBP-3 caused by EGF was highly reproducible and half maximal at 3 ng/ml **(Fig 3*b*)**. The slight reduction caused by bFGF was half maximal at 3 ng/ml **(Fig 3*c*)**, whereas the effect of retinoic acid was half maximal at approximately 10^{-8} M **(Fig 3*d*)**. Dexamethasone from 10^{-11} to 10^{-7} M caused changes of less than 10% in IGFBP-3 level **(Fig 3*e*)**.

EGF Reduces IGFBP-3 mRNA Levels in HaCaT Cells To investigate the mechanism behind the changes in IGFBP-3 levels in conditioned media caused by IGF-I and EGF, we measured the levels of IGFBP-3 mRNA in HaCaT cells after treatment with each hormone. HaCaT cells were incubated in growth factor–free DMEM for 24 h and then grown in the presence of each hormone for 2 d with daily changes of hormone in fresh medium. Total RNA was then prepared, and the relative levels of IGFBP-3 mRNA were determined by Northern analysis as described in *Materials and Methods*. As shown in **Fig 4*a***, IGF-I stimulation of IGFBP-3 was not accompanied by an increase in IGFBP-3 mRNA. Densitometric scanning of triplicate lanes (gel not shown) revealed that IGF-I in fact caused a slight (approximately 0.2-fold) reduction in IGFBP-3 mRNA band intensity **(Fig 4*b*)**. The reduction in IGFBP-3 levels after EGF treatment, however, was accompanied by a highly significant fivefold reduction in IGFBP-3 mRNA compared to control untreated cells **(Fig 4*b*)**.

DISCUSSION

We have investigated the regulation of IGFBP-3 production in HaCaT cells, a keratinocyte cell line exhibiting purely basal characteristics. Several hormones known to be important in the regulation of skin growth were tested, resulting in the following observations: i) IGF-I and EGF are potent mitogens of HaCaT cells, as they are of other keratinocyte cultures [2,12]; ii) IGF-I raises the level of IGFBP-3 in cell-conditioned medium by a mechanism that is not dependent on increases in IGFBP-3 mRNA; iii) EGF is a potent inhibitor of IGFBP-3 synthesis, significantly reducing its protein and mRNA level; iv) bFGF and retinoic acid inhibit IGFBP-3 production only slightly; and v) dexamethasone has no significant effects on IGFBP-3 level.

The effect of IGF-I on IGFBP production has been studied in several cell types. Receptor-mediated IGF-I stimulation of IGFBP-3 with concomitant increases in IGFBP-3 mRNA has been demonstrated in human bone cells and osteosarcomas [27] and a bovine kidney epithelial cell line [28]. Receptor-independent IGF-I stimulation of IGFBP-3 has been demonstrated in a human breast cancer cell line [29], human dermal fibroblasts [30,31], and a human squamous cell carcinoma line [31]. In dermal fibroblasts, the IGFBP-3 stimulation was shown to be independent of increased IGFBP-3 mRNA [32] and due to increased IGFBP-3 release [30,31]. From these studies and our findings presented here, it appears that both of the major cell types in skin, dermal fibroblasts and epidermal keratinocytes, share a similar mechanism of IGFBP-3 regulation by IGF-I. Whether this involves IGF-I–mediated release of IGFBP-3 from keratinocytes remains to be investigated, but by analogy with the similar IGF-I effect observed in the squamous cell carcinoma line [31], the IGF receptor is unlikely to be involved. Here we demonstrated that in HaCaT cells the effect is independent

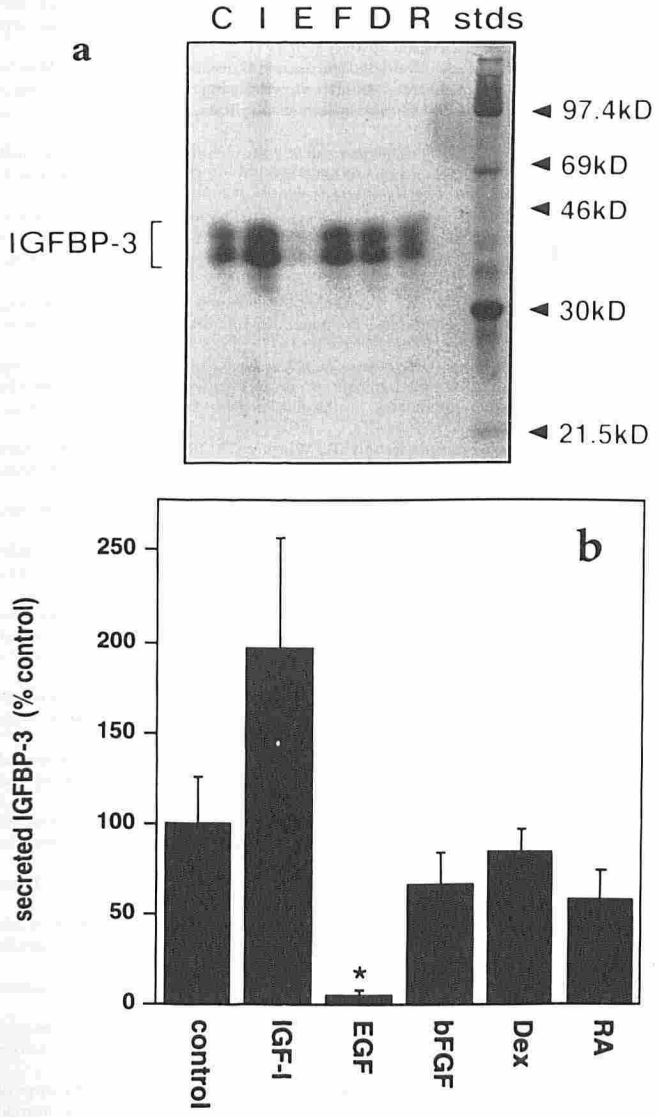

Figure 2. Hormonal effects on IGFBP-3 production in HaCaT cells. Post-confluent monolayers of HaCaT cells were treated with four daily additions of each hormone, then 2 d after the final addition IGFBP-3 in cell-conditioned media were analyzed by Western ligand blotting as in *Materials and Methods*. *a*, autoradiogram of Western ligand blot. C, control (untreated) cells; I, 100 ng/ml IGF-I; E, 30 ng/ml EGF; F, 30 ng/ml bFGF; D, 10^{-7} M dexamethasone; R, 10^{-6} M retinoic acid. *b*, scanning densitometry of Western ligand blot autoradiographs showing band intensities relative to control untreated cells after hormone treatment at the above concentrations. *Bars* represent the means of three separate experiments ± SD. *p < 0.001 versus control group.

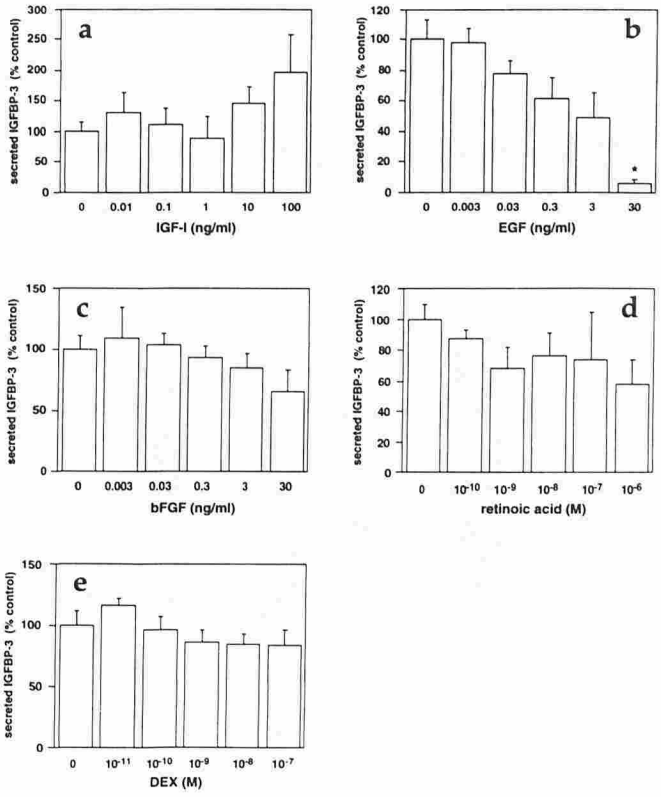

Figure 3. Concentration dependence of hormonal effects on IGFBP-3. Post-confluent monolayers of HaCaT cells were treated with four daily additions of each hormone at the concentrations indicated. Two days after the final addition, IGFBP-3 in the cell conditioned media was measured by scanning densitometry of Western ligand blots as in *Materials and Methods*. *Bars* represent the means of three separate experiments ± SD. *p < 0.001 versus control group.

of increases in IGFBP-3 mRNA and so probably does not require increased IGFBP-3 synthesis. IGF-I–mediated protection of released IGFBP-3 from proteolysis is also not likely to be involved, because in a previous study we found no such proteolytic activity in HaCaT-conditioned media [7].

In contrast to IGF-I, EGF appears to have opposing effects on IGFBP-3 production in keratinocytes and fibroblasts. In Swiss 3T3 cells, EGF stimulated IGFBP-3 levels in conditioned media [21], whereas in HaCaT cells we observed inhibition of IGFBP-3 synthesis using the same treatment protocol. There are other reports of IGFBP-3 production being stimulated by EGF, for example in porcine granulosa cells [33] and sheep thyroid cells [34], but the only other published example of EGF inhibition of IGFBP-3 production interestingly occurs in another epithelial cell line, one derived from murine mammary gland [35]. In HaCaT cells, the mechanism of inhibition of IGFBP-3 synthesis by EGF remains to be determined but appears to involve either inhibition of IGFBP-3 gene transcription or changes in IGFBP-3 mRNA stability, or both.

The regulation of IGFBP-3 in basal keratinocytes by IGF-I and EGF is significant for several reasons. Basal keratinocytes are the main epidermal targets for IGF-I and EGF, because IGF [5] and EGF [13,14] receptors are concentrated in the basal layer. IGFBP-3 secretion by basal keratinocytes does decrease their sensitivity to IGF-I [7], so, conversely, inhibition of IGFBP-3 synthesis by EGF could significantly increase their sensitivity to IGF-I, resulting in a synergistic effect of EGF and IGF on basal keratinocyte growth. One precedent for this type of growth-factor co-operation is the observation that tumor necrosis factor-α both inhibits IGFBP-3 production and increases IGF-I sensitivity in fibroblasts, whereas transforming growth factor-β1 has the opposite effects [36]. Synergism between IGF-I and EGF action has been demonstrated previously in keratinocytes, in which IGF-I up-regulates expression of the EGF receptor [37], and in fibroblasts, in which EGF stimulates IGF-I release in 3T3 cells in a possible autocrine loop [38]. The inhibition of IGFBP-3 synthesis through the EGF receptor on keratinocytes may be a further example of this synergism and may be especially significant in the hyperproliferation of basal keratinocytes occurring in psoriasis. In psoriasis, both the EGF receptor and its epidermally derived ligand, transforming growth factor-α, are overexpressed [39]. Based on our findings, we suspect that the resulting increase in stimulation of the EGF receptor is likely to result in suppression of IGFBP-3 in the basal epidermis. Thus, IGFBP-3 suppression, together with the overexpression of

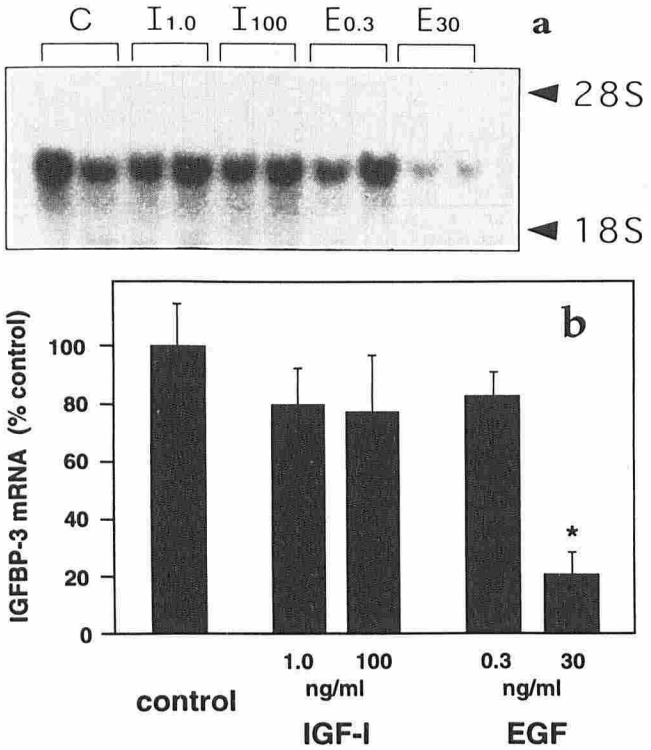

Figure 4. EGF causes a reduction of IGFBP-3 mRNA level in HaCaT cells. Post-confluent monolayers of HaCaT cells were treated with two daily additions of IGF-I or EGF at the concentrations indicated, and 20 μg total RNA from the treated cells was analyzed on a Northern blot probed with a ^{32}P-labeled human IGFBP-3 cDNA as in *Materials and Methods*. *a*, autoradiograph of Northern blot showing the 2.6-kb IGFBP-3 mRNA. C, control (untreated) cells; $I_{1.0}$, 1.0 ng/ml IGF-I; I_{100}, 100 ng/ml IGF-I; $E_{0.3}$, 0.3 ng/ml EGF; E_{30}, 30 ng/ml EGF; 28S, 18S, positions of 28S and 18S ribosomal RNAs as determined by ethidium bromide fluorescence of transferred RNA. *b*, scanning densitometry of Northern autoradiograms, performed in triplicate. The graph shows the optical densities of scanned triplicate IGFBP-3 mRNA bands, following hormone treatment at the above concentrations, relative to control, untreated samples. Relative optical densities were standardized against the optical density of the 18S ribosomal RNA band in each lane. *Bars* represent means of triplicate lanes ± SD. *$p < 0.02$ versus control group.

IGF-I receptors known to occur in psoriasis [5], is likely to result in significant IGF-I–driven keratinocyte proliferation. It is also possible that similar synergistic effects between mitogenic growth factors and IGFBP-3 increase IGF-I-driven hyperproliferation of basal keratinocytes in other epidermal diseases or during wound healing.

Because HaCaT cells are a spontaneously immortalized keratinocyte line, we cannot rule out the possibility that changes may have occurred in their regulation by growth factors. However, we have shown that their growth response, to EGF and IGF-I at least, is like that of normal cultured keratinocytes. To the extent that we can extrapolate our observations to keratinocytes *in vivo*, we conclude that EGF and IGF-I could alter the IGF-I sensitivity of the basal epidermis by regulating the production of the major IGF binding protein of the basal keratinocyte, IGFBP-3.

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REFERENCES

1. Ristow H-J, Messmer TO: Basic fibroblast growth factor and insulin-like growth factor I are strong mitogens for cultured mouse keratinocytes. *J Cell Physiol* 137:277–284, 1988
2. Neely EK, Morhenn VB, Hintz RL, Wilson DM, Rosenfeld RG: Insulin-like growth factors are mitogenic for human keratinocytes and a squamous cell carcinoma. *J Invest Dermatol* 96:104–110, 1991
3. Barreca A, deLuca M, del Monte P, Bondanza S, Damonte G, Cariola G, diMarco E, Giordano G, Cancedda R, Minuto F: In vitro paracrine regulation of human keratinocyte growth by fibroblast-derived insulin-like growth factors. *J Cell Physiol* 151:262–268, 1992
4. Liu J-P, Baker J, Perkins AS, Robertson EJ, Efstratiadis A: Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* 75:59–72, 1993
5. Krane JF, Gottlieb AB, Carter DM, Krueger JG: The insulin-like growth factor I receptor is over-expressed in psoriatic epidermis, but is differentially regulated from the epidermal growth factor receptor. *J Exp Med* 175:1081–1090, 1992
6. Boukamp P, Petrussevska RT, Breitkreuz D, Hornung J, Markham A, Fusenig NE: Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 106:761–771, 1988
7. Wraight CJ, Murashita MM, Russo VC, Werther GA: A keratinocyte cell line (HaCaT) synthesizes a predominant insulin-like growth factor binding protein (IGFBP-3) which modulates insulin-like growth factor-I action. *J Invest Dermatol* 103:627–631, 1994
8. Batch JA, Mercuri FA, Edmondson SR, Werther GA: Localization of messenger ribonucleic acid for insulin-like growth factor binding proteins in human skin by *in situ* hybridization. *J Clin Endocrinol Metab* 79:1444–1449, 1994
9. Clemmons DR: IGF Binding proteins: regulation of cellular actions. *Growth Reg* 2:80–87, 1992
10. Conover CA: Potentiation of insulin-like growth factor (IGF) action by IGF binding protein-3: studies of underlying mechanism. *Endocrinology* 130:3191–3199, 1992
11. Hamon GA, Hunt TK, Spencer EM: In vivo effects of systemic insulin-like growth factor-I alone and complexed with insulin-like growth factor binding protein-3 on corticosteroid suppressed wounds. *Growth Reg* 3:53–56, 1993
12. O'Keefe EJ, Chiu ML: Stimulation of thymidine incorporation in keratinocytes by insulin, epidermal growth factor, and placental extract: comparison with cell number to assess growth. *J Invest Dermatol* 90:2–7, 1988
13. Green MR, Couchman JR: Differences in human skin between the epidermal growth factor receptor distribution detected by EGF binding and monoclonal antibody recognition. *J Invest Dermatol* 85:239–245, 1985
14. King LE, Gates RE, Stoscheck CM, Nanney LB: Epidermal growth factor/transforming growth factor alpha receptors and psoriasis. *J Invest Dermatol* 95:10S–12S, 1990
15. O'Keefe EJ, Chiu ML, Payne RE: Stimulation of growth of keratinocytes by basic fibroblast growth factor. *J Invest Dermatol* 90:767–769, 1988
16. Shipley GD, Keeble WW, Hendrickson JE, Coffey RJ, Pittelkow MR: Growth of keratinocytes and fibroblasts in serum-free medium is stimulated by acidic and basic fibroblast growth factor. *J Cell Physiol* 138:511–518, 1989
17. Fuchs E, Green H: Regulation of terminal differentiation of cultured human keratinocytes by vitamin A. *Cell* 25:617–625, 1981
18. Kopan R, Traska G, Fuchs E: Retinoids as important regulators of terminal differentiation: examining keratin expression in individual cells at various stages of keratinization. *J Cell Biol* 105:427–440, 1987
19. Breitkreuz D, Stark HJ, Plein P, Baur M, Fusenig NE: Differential modulation of epidermal keratinization in immortalized (HaCaT) and tumorigenic human skin keratinocytes (HaCaT-ras) by retinoic acid and extracellular Ca^{2+}. *Differentiation* 54:201–217, 1993
20. Sheikh MS, Shao Z-M, Husain A, Clemmons DR, Roberts CT Jr, LeRoith D, Fontana JA: Regulation of insulin-like growth factor-binding protein-1, 2, 3, 4, 5 and 6: synthesis, secretion, and gene expression in estrogen receptor-negative human breast carcinoma cells. *J Cell Physiol* 155:556–567, 1993
21. Corps AN, Brown KD: Mitogens regulate the production of insulin-like growth factor-binding protein by Swiss 3T3 cells. *Endocrinology* 128:1057–1064, 1991
22. Shimasaki S, Ling N: Identification and molecular characterization of insulin-like growth factor binding proteins (IGFBP-1, -2, -3, -4, -5 and -6). *Prog Growth Factor Res* 3:243–266, 1991
23. Schulz J, Dettlaff S, Fritzsche U, Harms U, Schiebel H, Derer W, Fusenig NE, Hülsen A, Böhm M: The amido black assay: a simple and quantitative multipurpose test of adhesion, proliferation, and cytotoxicity in microplate cultures of keratinocytes (HaCaT) and other cell types growing adherently or in suspension. *J Immunol Meth* 167:1–13, 1994
24. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970
25. Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S, Binoux M: Analysis of serum insulin-like growth factor binding proteins using Western blotting: use of the method for titration of the binding proteins and competitive binding studies. *Anal Biochem* 154:138–143, 1986
26. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
27. Mohan S, Strong DD, Lempert UG, Tremollieres F, Wergedal JE, Baylink DJ (1992): Studies on regulation of insulin-like growth factor binding protein (IGFBP)-3 and IGFBP-4 production in human bone cells. *Acta Endocrinologica* 127:555–564, 1992
28. Cohick WS, Clemmons DR: Regulation of insulin-like growth factor binding protein synthesis and secretion in a bovine epithelial cell line. *Endocrinology* 129:1347–1354, 1991
29. Oh Y, Müller HL, Pham H, Lamson G, Rosenfeld RG: Non-receptor mediated,

post-transcriptional regulation of insulin-like growth factor binding protein (IGFBP)-3 in Hs578T human breast cancer cells. *Endocrinology* 131:3123–3125, 1992

30. Conover CA: A unique receptor-independent mechanism by which insulin-like growth factor I regulates the availability of insulin-like growth factor binding proteins in normal and transformed human fibroblasts. *J Clin Invest* 88:1354–1361, 1991
31. Neely EK, Rosenfeld RG: Insulin-like growth factors (IGFs) reduce IGF-binding protein-4 (IGFBP-4) concentration and stimulate IGFBP-3 independently of IGF receptors in human fibroblasts and epidermal cells. *Endocrinology* 130:985–993, 1992
32. Martin JL, Ballesteros M, Baxter RC: Insulin-like growth factor-I (IGF-I) and transforming growth factor-beta 1 release IGF-binding protein-3 from human fibroblasts by different mechanisms. *Endocrinology* 131:1703–1710, 1992
33. Mondschein JS, Smith SA, Hammond JM: Production of insulin-like growth factor binding proteins (IGFBPs) by porcine granulosa cells: identification of IGFBP-2 and -3 and regulation by hormones and growth factors. *Endocrinology* 127:2298–2306, 1990
34. Bachrach LK, Eggo MC, Burrow GN, Liu F, Tram T, Powell DR: Regulation of insulin-like growth factor-binding protein messenger ribonucleic acid levels in sheep thyroid cells. *Endocrinology* 128:1967–1973, 1991
35. Skaar TC, Baumrucker CR: Regulation of insulin-like growth factor binding protein secretion by a murine mammary epithelial cell line. *Exp Cell Res* 209:183–188, 1993
36. Yateman ME, Claffey DC, Cwyfan-Hughes SC, Frost VJ, Wass JA, Holly JM: Cytokines modulate the sensitivity of human fibroblasts to stimulation with insulin-like growth factor-I (IGF-I) by altering endogenous IGF-binding protein production. *J Endocrinol* 137:151–159, 1993
37. Krane JF, Murphy DP, Carter DM, Krueger JG: Synergistic effects of epidermal growth factor (EGF) and insulin-like growth factor I/somatomedin C (IGF-I) on keratinocyte proliferation may be modulated by IGF-I transmodulation of the EGF receptor. *J Invest Dermatol* 96:419–424, 1991
38. Pietrzkowski Z, Sell C, Lammers R, Ullrich A, Baserga R: Roles of insulinlike growth factor 1 (IGF-1) and the IGF-1 receptor in epidermal growth factor-stimulated growth of 3T3 cells. *Mol Cell Biol* 12:3883–3889, 1992
39. Krueger JG, Krane JF, Carter DM, Gottlieb AB: Role of growth factors, cytokines, and their receptors in the pathogenesis of psoriasis. *J Invest Dermatol* 94:135S–140S, 1990