Synergistic Effects of Epidermal Growth Factor (EGF) and Insulin-Like Growth Factor I/Somatomedin C (IGF-I) on Keratinocyte Proliferation May Be Mediated by IGF-I Transmodulation of the EGF Receptor

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The epidermal growth factor (EGF) receptor pathway is an important mediator of keratinocyte growth in vitro and both receptor and ligand components of this pathway are abnormally expressed in hyperproliferative epidermis. The purpose of this study was to examine interactions between the EGF receptor pathway and the insulin-like growth factor I/somatomedin C (IGF-I) receptor pathway in modulating the growth of cultured normal human keratinocytes. Short-term growth of keratinocytes in a chemically defined medium demonstrated that neither EGF nor IGF-I alone could support significant keratinocyte spreading or proliferation, but that a combination of EGF with IGF-I or high-dose insulin could. IGF-I or high-dose insulin transmodulates keratinocyte EGF receptor expression via the IGF-I receptor in a dose- and time-dependent manner, increasing EGF receptor binding an average of 1.8 times up to a maximum of fourfold without altering EGF binding affinity. Staining of normal human epidermis with an IGF-I receptor specific monoclonal antibody demonstrates that IGF-I receptors localize to the basal proliferative cell compartment, suggesting that IGF-I receptor and EGF receptor pathway interactions may play a role in the regulation of epidermal growth and in the pathogenesis of hyperproliferative skin diseases. *J Invest Dermatol 96:419–424, 1991*

Hormone-receptor systems permit extracellular molecules to control growth or metabolic cellular events in diverse cell types. Growth of connective tissue cells such as fibroblasts is thought to be regulated principally by four families of growth-inducing hormones (EGF, FGF, insulin-like factors, and PDGF), which bind to plasma membrane receptors and stimulate intrinsic tyrosine kinase activity of these receptors [1]. However, hormonal function may differ according to cell type and different epithelial cell types appear to have distinct growth factor requirements. In keratinocytes, unlike fibroblasts, PDGF is not a mitogen because keratinocytes lack PDGF receptors [2]. The other principal tyrosine kinase pathways controlling fibroblast growth also influence the growth of cultured keratinocytes. A mixture of EGF, insulin or IGF-I, and FGF or FGF-containing pituitary or brain extracts is required for the optimal growth of keratinocytes in serum-free medium [3–7]. Insulin/IGF-I is the only factor absolutely required to support keratinocyte proliferation; however, neither insulin/IGF-I nor any other growth factor is individually sufficient to promote clonal cell growth [8]. Comparatively little is known about the molecular pathways of growth or differentiation induced by activation of growth factor receptors in normal keratinocytes, or about possible interactions and potential synergies between the various hormonal pathways.

Control of keratinocyte growth via the EGF receptor pathway may be critical to the normal structure and function of the epidermis. Both EGF receptors and transforming growth factor-α (TGF-α), an EGF-related peptide, are detected in normal human epidermis, predominantly within the basal proliferative cell compartment [9,10]. Hyperplastic epidermis in psoriasis displays increased EGF receptors [11] and increased TGF-α [12,13], suggesting a potential role for this ligand-receptor system in keratinocyte growth regulation. However, the function of the EGF receptor system in human epidermis has not been precisely defined. As originally described in mouse experiments, EGF promoted skin maturation (eyelid opening) and produced increased thickness (acanthosis) of normal appearing epidermis [14,15]. The effects of EGF on human keratinocyte growth and maturation have been examined in a variety of serum-containing and serum-free tissue culture systems [3–5,16]. EGF promotes clonal or colony growth of human keratinocytes, extends the in vitro lifespan of cultured keratinocytes, and prevents terminal differentiation under some conditions [16]. Its effects in colony growth assays are related to effects on proliferation, cell cytoplasm size, and migration of keratinocytes [17,18]. However, EGF is not absolutely required for keratinocyte proliferation [4,6–8] and it appears to have a negligible effect on keratinocyte

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Abbreviations:
EGF: epidermal growth factor
FCS: fetal calf serum
FGF: fibroblast growth factor
HC: hydrocortisone
IGF-I: insulin-like growth factor I/somatomedin C
KBM: keratinocyte basal medium
KGM: keratinocyte growth medium
PDGF: platelet-derived growth factor
TGF-α: transforming growth factor-α

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proliferation in a number of assays [19,20]. Furthermore, a number of transformed keratinocyte lines that overexpress EGF receptors show clear dose-dependent inhibition of proliferation in response to EGF [19]. Because keratinocytes synthesize and release functional TGF-α [9], the role of exogenous EGF in keratinocyte proliferation may be obscured by potential effects of endogenous keratinocyte-derived TGF-α acting on EGF receptors in an autocrine or paracrine fashion.

As the precise function of individual growth factors in the regulation of keratinocyte proliferation has not been determined, there is a need to further define the effects of EGF and other growth factors on human keratinocytes under standardized, defined culture conditions. In this study, we report on the simultaneous requirement for EGF and IGF-I high-dose insulin to support human keratinocyte spreading and proliferation in chemically defined medium. IGF-I/high-dose insulin upregulates the EGF receptor and may thus regulate keratinocyte responsiveness to EGF receptor ligands, EGF and TGF-α. IGF-I receptors are found within the basal proliferative compartment of normal human epidermis, indicating that potential interactions between the IGF-I and EGF receptor pathways may be relevant to the in vivo state. These results suggest a possible molecular mechanism for growth factor synergies involved in keratinocyte proliferation.

MATERIALS AND METHODS

Keratinocyte Growth Assay Cultured neonatal human foreskin keratinocytes, grown as previously described [8] in keratinocyte growth medium (KGM, Clonetics Corp., San Diego, CA), were trypsinized and the trypsin was neutralized with DMEM/10% FCS. Cells were collected by centrifugation and resuspended in keratinocyte basal medium (KBM, Clonetics) containing 0.5 μg/ml hydrocortisone (HC, Clonetics) with or without added factors. Factors added were bovine insulin (5 μg/ml, Clonetics), human recombinant insulin (5μg/ml Humulin, Eli Lilly and Co., Indianapolis, IN), human recombinant IGF-I (10 or 100 ng/ml), and/or human recombinant EGF (10 or 100 ng/ml). Both human recombinant IGF-I and human recombinant EGF were produced by Chiron Corp., Emeryville, CA and were obtained from Dr. T. Kiorpes (Ethicon, Inc., Somerville, NJ). Cells were seeded at a density of 4000 cells/cm^2 in 25 cm^2 tissue culture flasks (Corning #25100). Cells seeded in this manner in KGM had a 30–70% plating efficiency after 1 d. After 6 d, duplicate cultures of each growth condition were trypsinized and counted in a Coulter counter.

Immunohistochemistry Immunoperoxidase staining was performed as described previously [12] using 3-amino-9-ethylcarbazole (Sigma Co., St. Louis, MO) as the developing reagent. A well-characterized mouse monoclonal antibody, αIR-3, specifically recognizing the IGF-I receptor [21], was obtained through Dr. Steven Jacobs (Wellcome Research Laboratories, Research Triangle Park, NC). Monoclonal antibody EGFR1 (Amersham Corp., Arlington Heights, IL) directed against the EGF receptor has been previously used to examine epidermal EGF receptor staining [10]. Both antibodies were used at an IgG concentration of 1 μg/ml and staining was compared with an IgG isotypic control.

EGF Receptor Binding Assay Cultured human neonatal foreskin keratinocytes were grown in KGM to approximately 90% confluence in 24-well tissue culture plates. Cells were then transferred to KBM for 24 h, followed by incubation with KBM alone or containing human recombinant or bovine insulin (5 μg/ml, except where indicated) or IGF-I for the indicated time. Plates were then put on ice, washed once with binding buffer (BB : KBM + 5 mg/ml BSA + 20 mM HEPES buffer, pH 7.3), followed by the addition of 0.25 ml/well of ^{125}I-EGF (Amersham; SA-100 μCi/μg) in BB to each well. ^{125}I-EGF was added at 1 ng/ml, except for Scatchard analysis, where radiolabeled EGF was added up to a maximum of 5 ng/ml and supplemented with unlabeled receptor-grade EGF (Collaborative Research, Inc., Bedford, MA) to a final total concentration of 20 ng/ml. Plates were transferred to a rocking incubator at 4°C for 6 h and then washed 4 times with BB. Cells were lysed in 0.1 M NaOH/0.1% Triton X-100 and counts were read in a gamma counter. Nonspecific binding was measured with a 1000 times excess of unlabeled EGF and did not exceed 5% of total binding. Values given are mean specific binding of triplicate measurements and are representative of results obtained from at least two different primary cell lines.

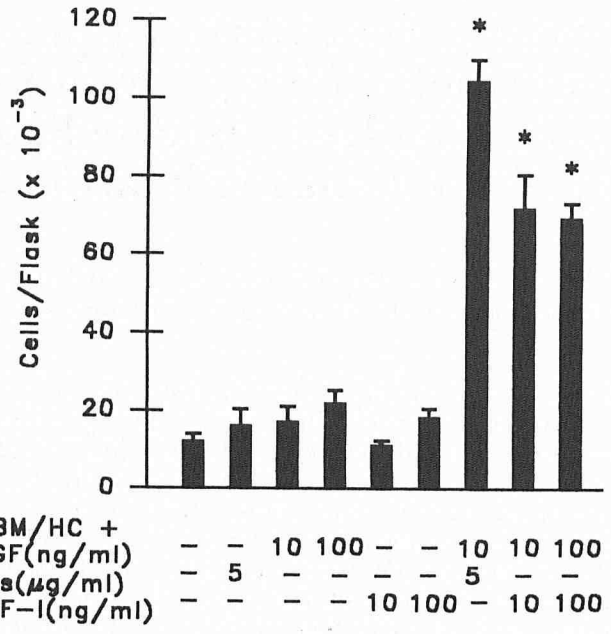

Figure 1. Keratinocyte growth factor studies. Keratinocytes were seeded as described in *Materials and Methods* in KBM/HC supplemented with human recombinant factors as indicated. After 6 d, cells were trypsinized and counted in a Coulter counter. Results represent the mean cell number of duplicate samples ± SD. The *asterisk* denotes a statistically significant ($p < 0.05$) difference compared to KBM/HC alone.

RESULTS

Keratinocyte Growth Requires EGF Combined with Either IGF-I or High-Dose Insulin We found that keratinocytes seeded in KGM and switched after 1 d to KBM continued to proliferate for at least 2 d, consistent with previous reports [22]. To assess the growth factor requirements of keratinocyte cultures, we therefore chose to seed keratinocytes in a minimal medium supplemented with growth factors individually or in combination, an approach used initially to define media requirements for clonal keratinocyte growth [4]. Figure 1 shows the results of such a growth experiment in which KBM containing 0.5 μg/ml HC was the minimal medium. Addition of insulin, IGF-I, or EGF as a single growth factor caused no statistically significant change in cell number after 6 d compared to control cultures. A marked stimulation of keratinocyte proliferation occurred when IGF-I or high-dose insulin was added in combination with EGF to the basal growth medium. Cells seeded in KBM/HC attached to the culture flask, but retained a rounded morphology for up to 6 d with no evidence of significant cell spreading. Cells seeded into medium containing EGF or high-dose insulin alone had a similar appearance to control at 1 and 6 d, although some EGF-treated cells spread and developed an epithelioid appearance by 6 d. A considerably enhanced degree of cell spreading was evident by 1 d after seeding with high-dose insulin and EGF. After an additional 5 d, typical colonies of proliferating keratinocytes were evident.

In these and all subsequent experiments, insulin was often substituted for IGF-I as previous work has indicated that the keratinocyte growth requirement for supraphysiologic insulin concentrations can be replaced by IGF-I [23] and is mediated through low-affinity insulin binding to and activation of the IGF-I receptor [24–26]. In all the experiments described below, IGF-I and high-dose insulin

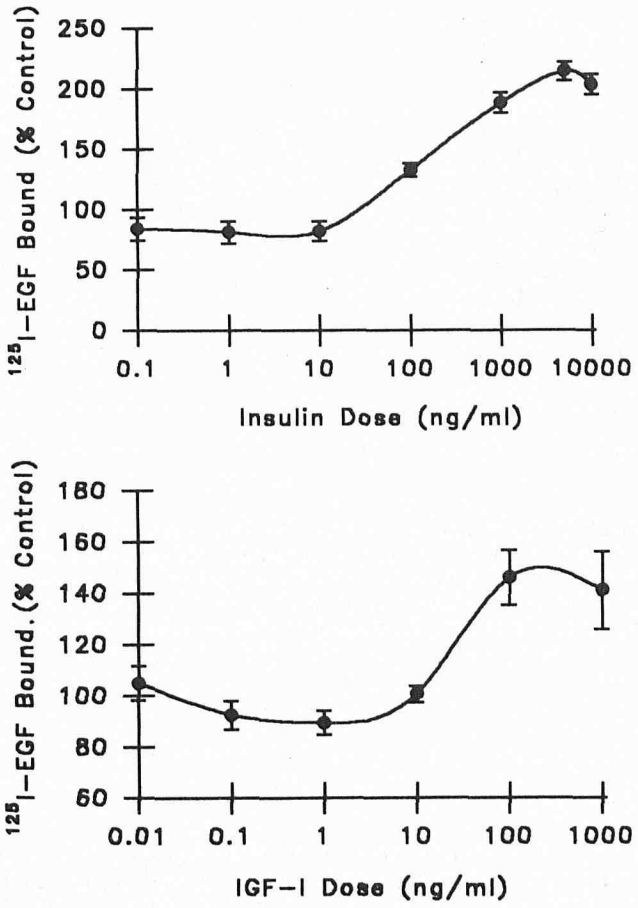

Figure 2. Dose-response of ^{125}I-EGF binding to keratinocytes treated with human recombinant insulin or IGF-I. Keratinocytes grown as described in *Materials and Methods* were treated for 2 d with the indicated concentrations of human recombinant insulin *(top)* or IGF-I *(bottom)*. Values were determined in triplicate and are expressed as the percent mean specific binding of cells in KBM alone. Primary keratinocyte lines derived from different individuals were used in the upper and lower parts of the figure.

had essentially the same effects on cultured keratinocytes. Thus, the results in Fig 1 indicate that activation of the EGF and IGF-I receptor systems together, but not either system individually, is sufficient to promote keratinocyte spreading and proliferation.

IGF-I or High-dose Insulin Increases EGF Binding to Keratinocytes Growth synergy between the IGF-I and EGF receptor systems might involve receptor "transmodulation," in which activation of one cytokine receptor pathway regulates another cytokine receptor pathway by altering receptor affinity, receptor number, and/or receptor tyrosine kinase activity. In order to assess IGF-I receptor mediated transmodulation of EGF receptor binding, we measured ^{125}I-EGF binding to the EGF receptor in cells that were first growth to near-confluence in KGM, then transferred for 24 h to KBM to allow "up-regulation" of cell surface EGF receptors [27], and finally incubated for 2 d with varying concentrations of insulin.

The upper graph in Fig 2 demonstrates a dose-dependent increase in binding of ^{125}I-EGF to keratinocytes treated with insulin. At low concentrations of insulin where the molecule binds predominantly to the insulin receptor [25], little or no decrease in EGF binding occurs. However, at supraphysiologic concentrations where insulin activates the IGF-I receptor [25], insulin increases EGF binding to a maximum of more than twofold in Fig 2 at an insulin concentration of 5 μg/ml. Carrier-free preparations of both bovine insulin and human recombinant insulin had similar effects on EGF receptor binding (data not shown). A half-maximal increase in EGF binding over untreated cells was calculated to occur at approximately 180 ng/ml insulin. This value correlates well with previous findings in which a half-maximal increase in mitogenicity in cultured keratinocytes occurred at an insulin concentration of 100–500 ng/ml [26,28]. This result is consistent with both the mitogenic and EGF receptor binding effects of high-dose insulin being mediated through the IGF-I receptor.

To confirm that high-dose insulin was acting through IGF-I receptors to increase binding of EGF to its receptor, we tested the effects of IGF-I on EGF receptor binding. The dose-response curve for EGF receptor binding in IGF-I–treated keratinocytes is shown in the lower graph in Fig 2. Like high-dose insulin, IGF-I causes a dose-dependent increase in ^{125}I-EGF binding to cultured keratinocytes. The dose-response for EGF receptor transmodulation coincides with previously described IGF-I–mediated keratinocyte proliferative responses [25]. In the example given, IGF-I increased EGF binding by a maximum of 1.5 times at a concentration of 100 ng/ml. Although the magnitude of the maximal increase shown in Fig 2 with IGF-I is less than that with insulin, this difference can probably be attributed to the use of different primary keratinocyte cell lines in the two experiments. The maximum stimulation of EGF binding varied slightly among cell lines, but ranged from approximately 1.5 to fourfold in five primary keratinocyte cell lines tested with an overall mean increase of 1.8 times. High-dose insulin and IGF-I have shown similar maximal effects on EGF binding when tested on the same cell line (data not shown). Some variability of response within cell lines also occurred, which may be partly attributable to the progressive down-regulation of cell-surface EGF receptors with time previously described in post-confluent terminally differentiating keratinocyte cultures [27].

Figure 3 shows EGF binding to keratinocytes using increasing ^{125}I-EGF concentrations and a Scatchard transformation of the data. Control keratinocytes grown in KBM for 2 d demonstrated a linear Scatchard plot at 4°C, indicating the presence of a single class of EGF receptors with a dissociation constant (K_d) of 1.8 nM. These cells possessed approximately 4.8×10^4 EGF binding sites/cell. Treatment for 2 d with 5 μg/ml human recombinant insulin resulted in no significant change in receptor binding affinity, but binding sites increased approximately 3 times to 17×10^4 sites/cell.

The increase in EGF binding caused by insulin was time dependent, as shown in Fig 4. Treatment of cells over a 48-h period with 5 μg/ml human recombinant insulin resulted in a continuous increase in EGF receptor binding. The greatest increase in EGF binding occurred within the first 12 h of treatment. Even a brief exposure to insulin of 1 min followed by 6 h at 4°C in binding medium free of insulin was sufficient to cause an increase in EGF binding in comparison with cells in KBM alone.

IGF-I Receptors are Expressed on the Surface of Basal Keratinocytes in Normal Human Epidermis Although the presence of IGF-I receptors on the surface of cultured human keratinocytes has been previously reported [24,25], the in vivo pattern of IGF-I receptor expression has not been described. In Fig 5*A,B*, the pattern of IGF-I receptor staining is shown in neonatal foreskin epidermis and in normal adult human epidermis using an IGF-I receptor specific mouse monoclonal antibody [21]. IGF-I receptors are expressed on the cell surface of keratinocytes in the basal, proliferative cell compartment of normal human epidermis. Staining of adult and neonatal epidermis was similar, although adult epidermis showed slightly weaker staining of the basal cell layer. An isotype-specific control antibody did not stain the epidermis (Fig 5C). An EGF receptor monoclonal antibody used at the same antibody concentration stained all viable epidermal layers (Fig 5D), in agreement with previous descriptions of EGF receptor distribution in normal human epidermis [10]. Thus, IGF-I receptors and EGF receptors are coexpressed on the surface of basal epidermal keratinocytes, where they could potentially act to regulate cell growth.

DISCUSSION

Our results indicate that IGF-I or insulin acts synergistically with EGF to promote keratinocyte spreading and growth in chemically defined MCDB 153 medium. IGF-I and insulin increase cell surface

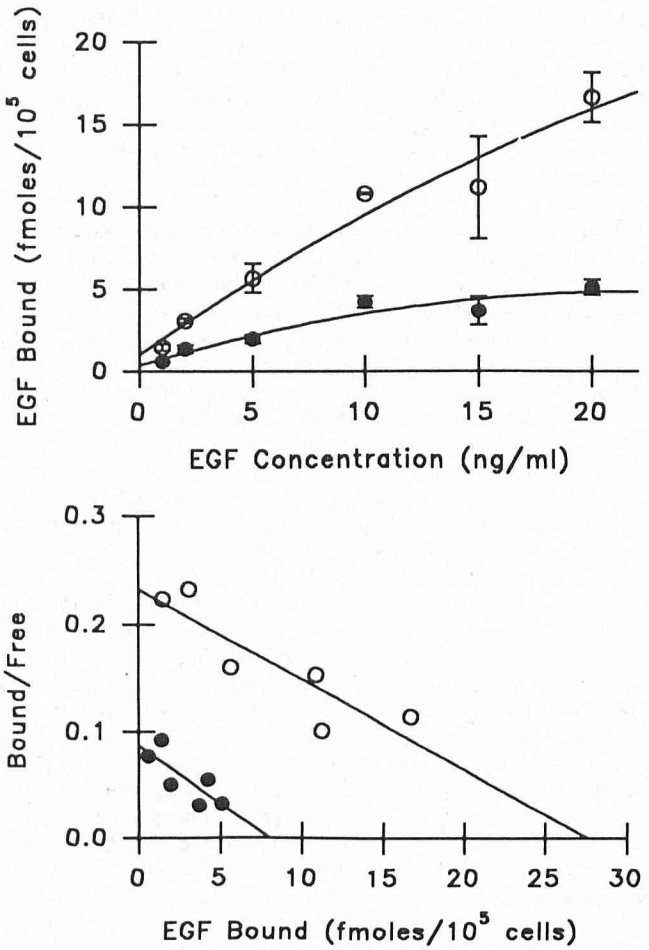

Figure 3. Scatchard analysis of ^{125}I-EGF binding to keratinocytes treated with human recombinant insulin. Binding was performed as described in *Materials and Methods* for cells treated 2 d with 5 μg/ml human recombinant insulin in KBM *(open circles)* or KBM alone *(closed circles)*. The upper part of the figure shows ligand binding with increasing concentrations of ^{125}I-EGF. The lower part of the figure is a Scatchard transformation of these data. Values given represent mean specific binding of triplicate measurements in a single experiment.

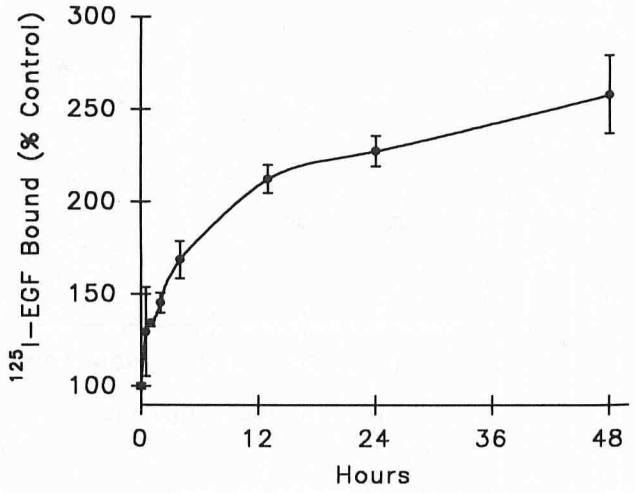

Figure 4. Kinetics of the increase in ^{125}I-EGF binding to keratinocytes treated with insulin. Keratinocytes were treated with 5 μg/ml human recombinant insulin in KBM as described in *Materials and Methods*. Early time points correspond to 0 min (no insulin addition), 1 min, 1 h, 2 h, and 4 h of insulin treatment. Values were determined in triplicate and are expressed as the percent mean specific binding of cells in KBM alone ± SD.

EGF receptors and this effect may be responsible, in part, for the observed synergism of EGF and insulin-like factors. Previous studies have demonstrated that clonal growth of human keratinocytes is dependent on EGF, insulin, and undefined growth factors present in pituitary extracts or bovine serum [8,16], but none have clearly demonstrated minimal growth factor requirements to sustain spreading and proliferation of human keratinocytes in chemically defined medium. Our data on human keratinocyte growth are in agreement with those on mouse keratinocytes, which demonstrate synergistic enhancement of DNA synthesis induced by IGF-I in combination with EGF or bFGF [26], or by insulin in combination with EGF [29].

At the low cell inoculation densities used in our studies, spreading and proliferation of human keratinocytes was dependent on the simultaneous presence of EGF and IGF-I/high-dose insulin. In a number of other studies, the effects of growth factors on keratinocyte proliferation have been measured after cell attachment and spreading is accomplished in a complete growth medium [20,25,30–32]. Under these conditions, growth responses to individual factors have been observed with less requirement for synergistic effects of EGF and IGF-I/insulin to support cell proliferation [20,25,30–32]. When we assayed the effects of EGF and IGF-I or insulin on keratinocyte growth after cells had attached and spread in complete growth medium, proliferation was no longer absolutely dependent on the simultaneous presence of EGF and insulin-like factors. Thus, part of the synergistic requirement for EGF and IGF-I/insulin may be related directly to effects on initial cell attachment and spreading. In this regard, keratinocytes treated with EGF and insulin increase synthesis of fibronectin 2–5 times in serum-free medium [31], and fibronectin or other connective tissue molecules can increase cell proliferation under these conditions [31,33].

IGF-I transmodulation of EGF receptors has not been previously described. In a transformed mouse keratinocyte line in which IGF-I and EGF growth synergy was noted, high-dose insulin had no effect on EGF receptor binding [29]. This cell line is strictly EGF dependent for growth in a serum-containing medium and expresses fewer EGF receptors than the normal human keratinocytes grown in serum-free medium, used in our experiments. Studies of EGF receptor binding in normal human keratinocytes show that the antiproliferative agents gamma interferon, transforming growth factor beta, and cyclosporin A decrease EGF receptor binding to 25–50% of untreated control levels [34]. Thus, our finding that the proliferative agents IGF-I and insulin increase EGF receptor binding is consistent with a model in which some molecules are capable of exerting stimulatory or inhibitory effects on keratinocyte growth via their ability to up- or down-regulate EGF receptor binding sites.

Synergistic effects between the IGF-I and EGF receptor pathways may also involve regulation of different aspects of cell cycle-mediated growth. In fibroblasts, insulin-like factors and EGF are necessary for mitogenic responses to PDGF but are poor intrinsic mitogens in the absence of PDGF (reviewed in [1]). Based on these observations, it has been postulated that growth factors act at specific, sequential points in the cell cycle to allow continued growth. In fibroblasts, PDGF is a major mitogen "competence" factor that must be present before EGF or insulin-like "progression" factors are able to stimulate proliferation via their effects on a later G_1 growth control point. Because keratinocytes lack PDGF receptors and are thus not directly responsive to PDGF [2], growth of connective tissue and epithelial cell types may be regulated by fundamentally different sets of growth factors and intracellular signaling pathways.

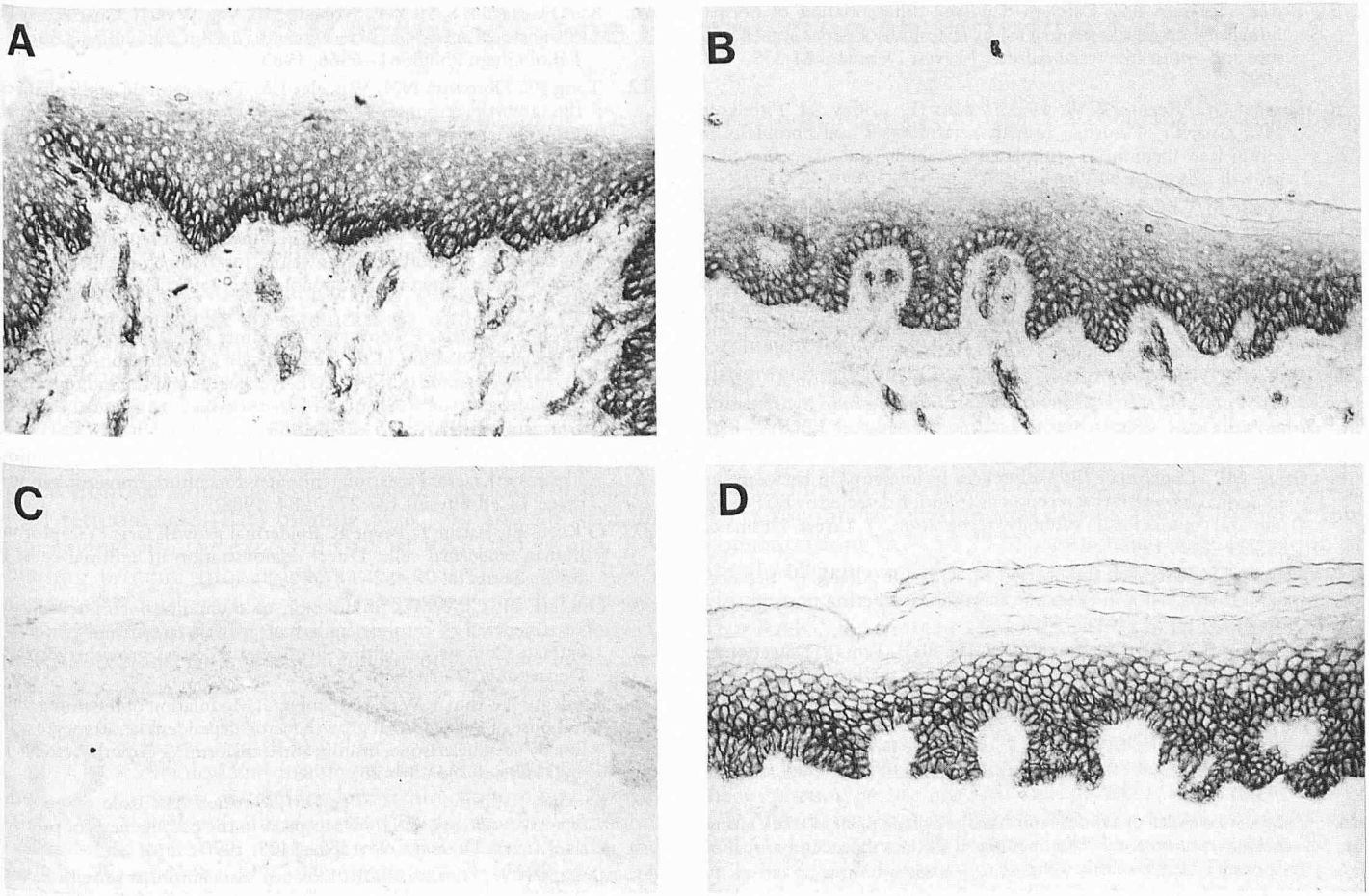

Figure 5. Staining of normal human epidermis with monoclonal antibodies against the IGF-I receptor and the EGF receptor by the immunoperoxidase technique. Sections of neonatal foreskin *(A)* and normal adult human skin *(B–D)* were processed as described in *Materials and Methods* using antibodies directed against the IGF-I receptor *(A,B)*, the EGF receptor *(D)*, and a non-specific isotype control *(C)*.

Insulin-like factors appear to be obligate for keratinocyte colony growth, but only if EGF, FGF, or a pituitary extract is simultaneously present [6–8]. IGF-I may act as a progression-like factor because it can stimulate proliferation of keratinocytes previously exposed to serum [25]. If IGF-I is in fact a progression factor in keratinocytes, then the growth factor synergy of IGF-I and EGF may result from EGF acting at another point in the cell cycle, perhaps serving as a competence factor.

Our staining data indicate that IGF-I receptors localize to the proliferative basal layer in normal epidermis, supporting the notion that interactions between the EGF and IGF-I receptor pathways could serve to modulate normal epidermal growth in vivo. Interactions between the EGF and IGF-I receptor pathways may also be relevant to pathologic growth states of the skin. The IGF-I receptor pathway may be involved in growth activation of psoriatic epidermis [24,25], in that somatostatin, which inhibits IGF-I production, may have some therapeutic effect on psoriasis [35]. Immunohistochemical staining for IGF-I receptors in psoriatic epidermis indicates that IGF-I receptor expression extends to suprabasal keratinocytes in psoriatic lesions.* Expansion of IGF-I receptor and EGF receptor expression beyond the basal cell layer may contribute to the increase in size of the proliferative cell compartment in psoriasis. Additionally, increased suprabasal IGF-I receptor activation may result in transmodulation of EGF receptors, which could contribute to the increase in EGF receptor binding described in psoriasis [11].

Cultured keratinocytes are subject to growth modification by numerous growth factors and cytokines in addition to those studied here, including FGF-related molecules and interleukins such as IL-1, IL-3, and IL-6 [6,7,20,30–32]. Which of these many growth factors is most important in regulating epidermal growth in vivo has not been determined. Multiple systems interacting in a complex manner may regulate growth of both normal and hyperproliferative skin. Future study of growth factor synergies and "cross-talk" between receptor systems in keratinocytes should provide a basis for gaining further insights into normal keratinocyte growth regulation, growth activation, and pathologic growth states.

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