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The Role of Tyrosine Kinase Growth Factor Receptors in Psoriatic Epidermal Hyperplasia: Analysis of Growth Factor Synergy in Cultured Human Keratinocytes and Receptor Expression in Psoriatic Skin

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The Role of Tyrosine Kinase Growth Factor Receptors in Psoriatic Epidermal Hyperplasia: Analysis of Growth Factor Synergy in Cultured Human Keratinocytes and Receptor Expression in Psoriatic Skin

A thesis submitted to the Faculty of The Rockefeller University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

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To My Parents with Love and Gratitude

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Abbreviations

aFGF	acidic fibroblast growth factor
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CSA	cyclosporin A
d	day(s)
DMEM	Dulbecco's modified Eagle's Medium
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
EGF	epidermal growth factor
FGF	fibroblast growth factor
GM-CSF	granulocyte and macrophage colony-stimulating factor
h	hour(s)
HC	hydrocortisone
HEPES	4-(2-hydroxyethyl)-1-piperazine ethansulfonic acid
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon gamma
IGF-I	insulin-like growth factor I/ somatomedin C
IL	interleukin
kb	kilobase(s)
KBM	Keratinocyte Basal Medium
kd	kilodalton(s)
K_d	dissociation constant
KGF	keratinocyte growth factor
KGM	Keratinocyte Growth Medium
mAb	monoclonal antibody
min	minute(s)
M_r	molecular radius or size
mRNA	messenger ribonucleic acid
NP-40	Nonidet P-40
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PHA	phytohaemagglutinin
PKC	protein kinase C
PMA	12-O-tetradecanoylphorbol-13-myristate
PMSF	phenylmethyl sulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TGF	transforming growth factor
TNF	tumor necrosis factor
TX-100	Triton X-100

Abstract

Psoriasis is a hyperproliferative disease of the skin characterized histologically by epidermal and dermal hyperplasia and inflammatory cell infiltrates. While the pathogenesis of psoriasis is unknown, altered expression of cytokine pathways is likely to produce many of the phenotypic changes in the disease. The epidermal growth factor (EGF) receptor pathway is an important mediator of keratinocyte growth and both ligand and receptor components of this pathway are abnormally expressed in hyperproliferative epidermis. In contrast, little is known about the function of other growth factor pathways in regulating keratinocyte growth. The purpose of this thesis was to characterize the function of three other major tyrosine kinase growth factor receptor pathways -- insulin-like growth factor I (IGF-I), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF), in the regulation of keratinocyte growth *in vitro* and to characterize the expression of these receptors in normal and psoriatic skin.

Growth of normal human keratinocytes in a chemically defined medium demonstrated that IGF-I, EGF, or basic FGF (bFGF) alone did not support significant keratinocyte spreading or proliferation. Considerable proliferation was observed when IGF-I was added in combination with either EGF or bFGF. In contrast, the combination of EGF with bFGF did not stimulate growth more than the addition of these factors alone. Growth synergy between IGF-I and EGF may be due in part to an IGF-I receptor mediated increase in keratinocyte EGF receptor expression. Treatment of cultured normal human keratinocytes with IGF-I increased EGF receptor binding an average of 1.8-fold in a dose and time dependent manner without altering EGF binding affinity.

To analyze potential growth factor pathway interactions *in vivo,* sections of normal and psoriatic human epidermis were stained with IGF-I receptor, EGF receptor, and FGF receptor specific antibodies. In normal skin, plasma membrane IGF-I receptor staining

localized exclusively to the proliferative basal cell compartment of the epidermis. IGF-I receptor staining was seen in both basal and suprabasal keratinocytes of lesional psoriatic skin correlating with the increased size of the proliferative cell compartment in psoriatic epidermis. In contrast, both EGF and FGF receptor expression was detected throughout the viable cell layers of the epidermis. Colocalization of IGF-I receptors with EGF and FGF receptors in proliferating epidermal keratinocytes suggests that interactions among growth factor pathways may be necessary for keratinocyte growth activation.

Since bFGF may regulate keratinocyte proliferation in skin, identification of a FGF receptor in keratinocytes was undertaken. Chemical crosslinking and immunoblotting experiments identified a single major FGF receptor protein with a molecular size of approximately 160 kd. Taken together with growth studies and immunohistochemical analysis, these data suggest that the FGF receptor protein identified may be involved in keratinocyte growth regulation.

PDGF is a potent mitogenic and chemotactic factor for fibroblasts and other cell types. Analysis of PDGF receptor metabolism and immunohistochemical staining indicated that keratinocytes do not express PDGF receptors and are thus not directly responsive to this hormone. However, expression of PDGF receptors was greatly elevated in dermal fibroblasts and blood vessels of growth-activated skin and could contribute to dermal hyperproliferative changes seen in psoriasis.

To begin analyzing the mechanism of action of anti-psoriatic drugs, the effects of anthralin and cyclosporin A (CSA) on keratinocyte proliferation and the EGF receptor pathway were examined. In contrast to CSA, anthralin-treated keratinocytes were more sensitive than lymphocytes to growth inhibition. CSA produced a cell cycle specific block at G_1 in keratinocytes, while anthralin did not specifically block the cell cycle at any stage. While CSA did not significantly decrease expression of the EGF receptor or its ligand, transforming growth factor-alpha (TGF-α), anthralin decreased both TGF-α mRNA levels and EGF receptor binding in cultured keratinocytes. TGF-α expression remained at high

levels in CSA-treated psoriasis patients suggesting that CSA does not act via direct modification of the EGF receptor pathway *in vivo*. These results may help explain differing response patterns to treatment with these two drugs.

The findings of this thesis suggest possible mechanisms of interaction among the pathways studied that might contribute to the psoriatic phenotype. In particular, IGF-I receptor expression may define keratinocyte proliferative potential, and may, therefore, be a promising target for the development of new anti-psoriatic therapies.

Chapter 1: Introduction

Much of what is known about growth regulation of human cells has been derived from *in vitro* studies of fibroblast cell lines. However, the vast majority of disorders of cell growth are of epithelial cell origin. The epidermis of the skin is a particularly suitable subject for the study of epithelial cell growth regulation. The stratified epithelium of the epidermis is composed predominantly of one cell type, the keratinocyte, which is readily accessible for study and can be successfully grown *in vitro.* Moreover, keratinocyte hyperproliferative states constitute a diverse and common group of conditions encompassing reversible hyperplasias such as normal wound healing and psoriasis as well as benign and malignant tumors. While the reversible nature of the psoriatic lesion is a key distinguishing trait from neoplastic epidermal growth, this feature provides the unusual opportunity to study both how growth regulatory mechanisms go awry as well as the methods by which proliferative homeostasis might be restored.

Psoriasis is a hyperproliferative disease of the skin with the clinical presentation of thick, erythematous scales that can vary greatly in distribution and appearance. The disease has a widespread prevalence affecting 1-2% of the United States population at an estimated cost of at least 1.5 billion dollars annually (1). Psoriasis, which is accompanied by severe arthritis in approximately 7% of patients (2), is often debilitating and in its most severe cases can even be life threatening. The disease is usually chronic and remitting in nature with precipitating factors including stress, certain drugs (such as lithium and β-blockers), and AIDS (3). Outbreaks of psoriasis have also been noted to occur at sites of prior skin injury (the Koebner phenomenon) (3). Genetic studies have demonstrated associations between major histocompatability antigens and psoriasis, particularly the Cw6 locus (4). Approximately one-third of psoriatics report a history of a first degree relative with the disease (4). Studies of monozygotic twins have shown a concordance for the

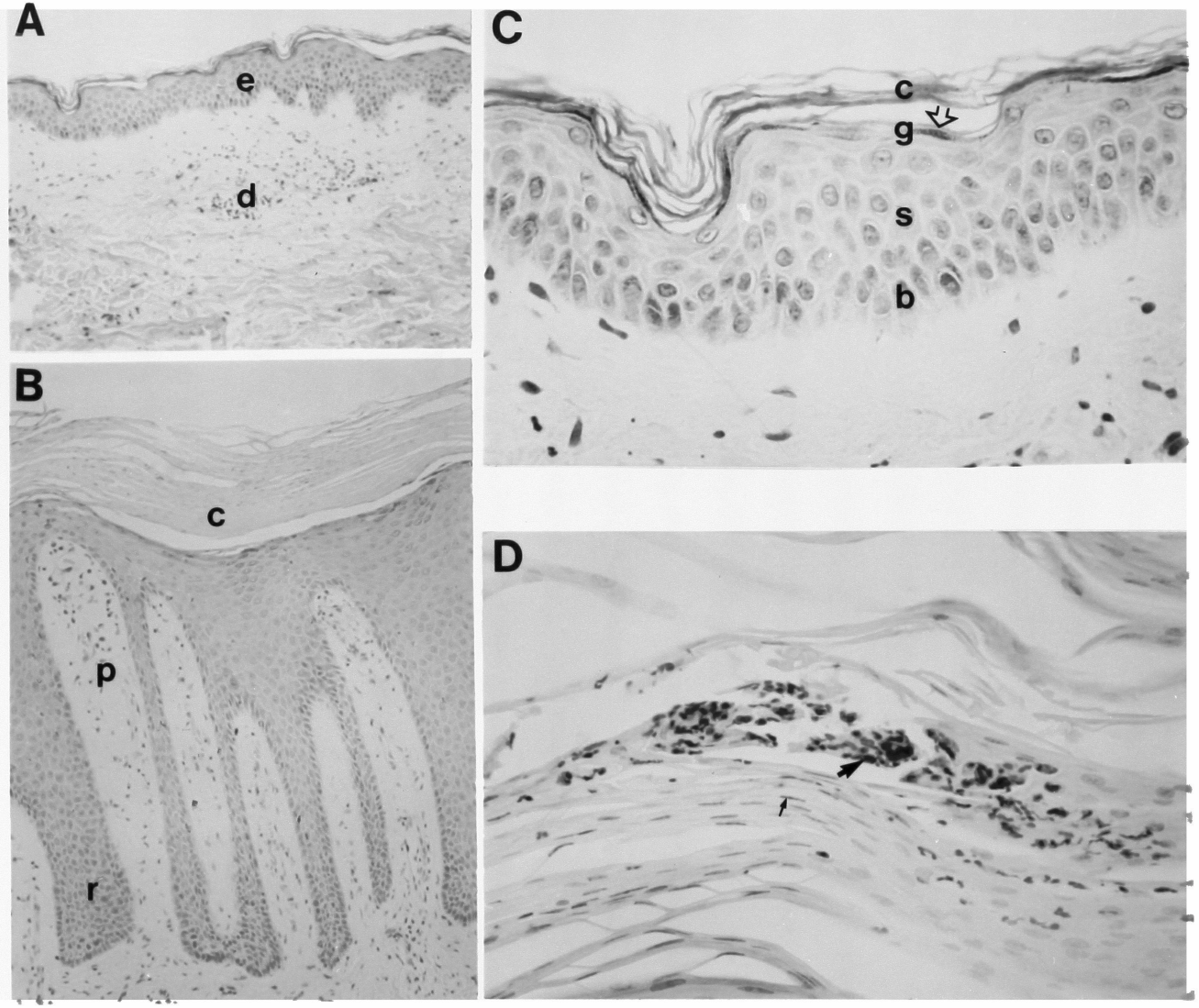
disease of 65-70% (4). On the basis of these studies as well as epidemiological data, it has been proposed that psoriasis is inherited as an autosomal dominant trait with approximately 60% phenotypic penetrance (3). It is unclear whether multiple genes are involved in transmission of the psoriatic genotype.

Histologically, psoriasis is characterized by dermal and epidermal hyperplasia accompanied by inflammatory infiltrates of both neutrophils and mononuclear cells into the dermis and epidermis (see Figure 1). Epidermal hyperplasia (acanthosis), hyperkeratosis (hyperplasia of the stratum corneum), parakeratosis (retention of nuclei in the stratum corneum), and elongation of rete pegs are typical features and neutrophilic microabscesses may also be noted in the epidermis. Dermal changes include fibroblast hyperplasia, lengthening of the dermal papillae, chronic inflammatory cell infiltrates in the papillary dermis, and dilatation of capillaries within dermal papillae.

Ever since the initial pathologic descriptions of the hyperplastic and inflammatory components of psoriasis, theories regarding the etiology of psoriasis have differed over which component constitutes the primary lesion of the disease and which represents a secondary response. However, little experimental evidence existed to support any theory until very recently. Studies of the pathogenesis of psoriasis have been hampered by the lack of suitable animal models for the disease and the inability to reproduce in culture the complex variety of dermal/epidermal interactions observed *in vivo*. Therefore, most of what is currently known about the pathogenesis of psoriasis has been obtained through the study of tissue obtained from human subjects.

Early reports of increased mitotic figures in the basal and suprabasal layers of the epidermis highlighted the importance of epidermal growth activation in the pathogenesis of psoriasis (5). In 1963, Van Scott and Ekel (6) quantitated mitotic figures in lesional and nonlesional psoriatic skin from which they estimated that psoriatic epidermis turns over in 4 days instead of the usual 4 weeks. They also noted that the size of the proliferative cell compartment in psoriatic epidermis is approximately doubled, with

Figure 1. Histological sections of normal and psoriatic human skin. All sections are stained with hematoxylin and eosin. (A) Human skin consists of the external epidermis (e) formed by a keratinizing stratified squamous epithelium and the underlying dermis (d) providing a supportive connective tissue matrix and vascular supply to the epidermis. A dermal inflammatory infiltrate can be seen in this section which was taken from an individual exhibiting a fixed drug eruption. 10X. (B) Psoriatic skin in which epidermal hyperplasia can be seen. Epidermal thickening (acanthosis), thickening of the outer cornified layer (hyperkeratosis), and retention of nuclei in the cornified layer (parakeratosis) are all present. Note the prominent rete pegs (r) as well as the elongated dermal papillae (p) containing dilated capillaries. Some chronic inflammatory cells can also be seen in the dermis. Note that (A) and (B) are at the same magnification. 10X. (C) Normal skin in which the layers of the epidermis can be clearly distinguished. The basal layer (b) is a monolayer of keratinocytes in which the pool of proliferative cells reside. The more differentiated spinous layer (s) contains large, keratinizing cells, while the flattened cells of the granular layer (g) contain basophilic keratohyaline granules (open arrow). Finally, anucleate, keratin-filled, cells form the outermost protective cornified layer (c) of the skin. Note the contrast in thickness of the stratum corneum in normal skin versus psoriatic skin in (B). 40X. (D) Stratum corneum of psoriatic skin in which a neutrophilic microabscess can be seen (large arrow) as well as nuclear retention within cornified cells (small arrow). 40X.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numerous mitoses found in both the suprabasal and basal layers of the epidermis. Subsequent study has estimated that keratinocyte cell cycling time is accelerated approximately 10-fold in psoriatic lesions (7). Additionally, psoriatic epidermis has been shown to share many features with epidermal changes observed in the wound healing process, which have been collectively termed "regenerative maturation" (8). However, simple hyperproliferation of the epidermis cannot account for all the characteristics of chronic immune activation in psoriatic skin including the identification of dermal infiltrates of Langerhans cells and activated T-cells as a prominent feature of the psoriatic lesion (9-11). Features similar to psoriasis can be produced by positive tuberculin skin tests or intradermal injection of the activated T cell product, IFN-γ (12,13). These findings have led to the hypothesis that the epidermal hyperproliferation of psoriasis may be mediated by immunological processes (10). Nevertheless, the identity of the primary defect in psoriasis remains uncertain; keratinocytes, Langerhans cells, T-cells, macrophages, endothelial cells, and fibroblasts have all been proposed as candidate initiators of the psoriatic phenotype. Even without definitive knowledge of the origin of the disease, research on psoriasis has made great strides with increasing attention focused on the evolution of the psoriatic lesion. Specifically, greater recognition is being given to the likelihood that cytokine production by some or all of the cell types mentioned above could mediate a complex array of interactions among the cells found in the skin, including immune activation, the recruitment of cells into the evolving psoriatic lesion, and mitogenic activation of dermal and epidermal cells.

Increased expression or altered regulation of growth-promoting cytokines and their receptors may play an important role in the pathogenesis of epidermal hyperplasia in psoriasis. It is therefore essential to identify specific growth factors or growth-promoting cytokines that regulate the proliferation of normal keratinocytes and which could be involved in the pathogenesis of psoriasis through aberrant expression. The development of adequate techniques for culturing human keratinocytes along with the recent availability

of specific monoclonal and polyclonal antibodies to cytokines and their receptors has made progress in these areas possible.

The ability to study keratinocyte proliferative requirements did not exist before 1975 when Rheinwald and Green (14) reported the first successful system for serially cultivating human keratinocytes. The system they described required the use of a feeder layer of mouse 3T3 cells as well as the presence of 20% fetal calf serum, thus making the analysis of the roles of different factors in stimulating keratinocyte growth difficult. Subsequent work by Peehl and Ham resulted in the development of a keratinocyte growth system that no longer required the feeder layer of cells and in which dialyzed serum replaced whole serum (15). Further optimization of the basic components of this medium resulted in the formulation of a serum-free medium termed MCDB153 (16), which supports clonal proliferation of keratinocytes when optimally supplemented with EGF, high concentrations of insulin, hydrocortisone, and bovine pituitary extract.

Advances in keratinocyte culture techniques as well as the development of specific antibodies to a variety of cytokines and their receptors have led to the identification of numerous cytokine pathways that could potentially be involved in the regulation of normal and psoriatic keratinocyte growth. Recent studies have demonstrated that psoriatic keratinocytes express HLA-DR, γ-IP-10, and ICAM-1, immune-related molecules which are not produced by keratinocytes in normal skin (9,17,18). HLA-DR and ICAM-1 regulate antigen presentation by and lymphocyte adherence to immune-derived cells and might serve an equivalent function in psoriatic epidermis (10,18). Gamma-IP-10 is a cytokine believed to have mitogenic and chemotactic properties, but its function in human skin is not known (17). Expression of these molecules could potentially stimulate a local immune reaction and provide signals for leukocyte migration into psoriatic epidermis. Synthesis of HLA-DR, γ-IP-10, and ICAM-1 by cultured keratinocytes can be induced by IFN-γ (17-19). IFN-γ has been detected in psoriatic epidermis leading to the proposal that activated T cells could induce keratinocyte production of HLA-DR, γ-IP-10, and ICAM-

1, further enhancing the immune response and epidermal leukocyte accumulation (20). However, these findings cannot fully explain the profound epidermal hyperplasia of psoriasis. IFN-γ has complex effects on keratinocyte growth, inhibiting keratinocyte proliferation *in vitro* (21) while causing epidermal hyperplasia and other psoriasiform changes when injected intradermally *in vivo* (13,22). Moreover, altered expression of many other cytokine pathways has been detected in psoriatic skin.

Normal cultured keratinocytes synthesize many cytokines including TGF-α, TGF-β, bFGF, IL-1, IL-3, IL-6, IL-8, and GM-CSF (23). Several of these factors stimulate the proliferation of cultured keratinocytes and might be involved in the pathogenesis of epidermal hyperplasia in psoriasis (24-27). Some of these cytokines might also promote immune activation or leukocyte accumulation in psoriasis since they also mediate functions of multiple immune-derived cell types. IL-1 and IL-6 stimulate growth of keratinocytes *in vitro* (20,24,25) and expression of both has been examined in psoriatic tissue (25,28). IL-6 levels are increased in psoriatic epidermis indicating that this protein may contribute to psoriatic epidermal hyperplasia (25). In contrast, IL-1 bioactivity in psoriatic epidermis is less than that found in normal skin (28).

Interpretation of findings regarding cytokine abnormalities in psoriatic skin is limited by our poor understanding of normal keratinocyte growth regulatory mechanisms. 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 (29). However, hormonal function may differ according to cell type and different epithelial cell types appear to have distinct growth factor requirements. While PDGF does not appear to be a mitogen in keratinocytes (23,30), 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 (16,31-34). 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 (35).

When this project was begun, the EGF receptor pathway was the only one of the four major tyrosine kinase pathways clearly implicated both in the control of keratinocyte growth in culture and in the maintenance of normal structure and function in human epidermis. As originally described in mouse experiments, EGF promoted skin maturation (eyelid opening) and produced acanthosis of normal appearing epidermis (36,37). 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 (14,16,31,32,38). Studies by Rheinwald and Green in a serum-containing system indicated that EGF promotes clonal or colony growth of human keratinocytes, extends the *in vitro* lifespan of cultured keratinocytes, and prevents terminal differentiation under some conditions (38). EGF effects in colony growth assays are related to effects on proliferation, cell cytoplasm size, and migration of keratinocytes (39,40). However, EGF is not absolutely required for keratinocyte proliferation (32,33,35) and it appears to have a negligible effect on keratinocyte proliferation in a number of assays (24,41). Furthermore, a number of transformed keratinocyte lines which overexpress EGF receptors, show clear dose-dependent inhibition of proliferation in response to EGF (41).

TGF-α, a peptide homologous to EGF, has similar biological effects to EGF (39) and appears to be the endogenous ligand for the EGF receptor in human skin (42). Since keratinocytes synthesize and release functional TGF-α (42), the molecule is an excellent candidate for an autocrine regulator of epidermal proliferation. TGF-α mRNA synthesis has been reported to be stimulated by a number of factors including EGF, TGF-α, IFN-γ, or phorbol ester treatment of keratinocytes (42-44). Increased TGF-α production is one mechanism by which phorbol ester or IFN-γ induced epidermal hyperplasia might occur (45). Immunohistochemical studies and mRNA analysis have detected TGF-α in the

epidermis of normal and psoriatic skin (26,27,42). Psoriatic keratinocytes in upper spinous layers show strong staining for TGF-α compared to the predominant staining of basal and lower spinous keratinocytes in normal-appearing skin of psoriatics or of normal human skin (26). Although TGF-α is also detected in the dermis of lesional skin, transcriptional analysis of TGF-α mRNA expression suggests that a substantial portion of TGF-α in psoriatic epidermis is produced by affected keratinocytes (27). Direct mitogenic stimulation of psoriatic keratinocytes by TGF-α has not been conclusively demonstrated and its ability to induce psoriasiform epidermal hyperplasia in human skin is also unproved.

While numerous cytokine abnormalities have been described in psoriasis, it has also been proposed that the level of expression of the cognate receptor for a cytokine can influence the degree of activation of a particular pathway (46). Overexpression of EGF receptors in 3T3 cells can cause cellular transformation, but only with the addition of low concentrations of ligand (47). Conversely, the lack of expression of a particular receptor may be necessary for proper cellular targeting of cytokines. In the skin, keratinocyte-derived PDGF (23) may act as a paracrine growth modulator of dermal fibroblasts without stimulating keratinocyte growth, since keratinocytes do not have PDGF receptors (23,30).

The EGF receptor is the only cytokine receptor to have been studied in detail in psoriasis. EGF receptors, like the EGF receptor ligand TGF-α, are overexpressed in hyperplastic psoriatic epithelium as determined by binding of ^{125}I-EGF to psoriatic skin (48). The overexpression of the EGF receptor and its ligand TGF-α in psoriatic skin suggests an obvious mechanism for increased keratinocyte proliferation in psoriasis. However, several observations suggest that the overexpression of TGF-α and the EGF receptor in psoriasis are not the sole causes of epidermal hyperplasia or of psoriasis. Increased expression of TGF-α and EGF receptors is seen in epidermal hyperplasias, such as wound healing, unrelated to psoriasis by clinical or histopathological criteria (20). Injection of EGF into the dermis of mouse skin produces epidermal acanthosis in a non-psoriasiform pattern without keratinocyte parakeratosis (37). Overexpression of TGF-α

in epidermal tissue of transgenic mice has been noted to produce focal epidermal hyperplasia, though a second stimulus may be required for extensive skin involvement (49). Taken as a whole, these data do suggest that the EGF receptor pathway could regulate at least some features of epidermal hyperplasia seen in psoriasis.

Interaction of TGF-α with abundant EGF receptors in psoriasis could explain numerous biochemical alterations detected in psoriatic skin. Molecular events associated with EGF induced mitogenic signaling have been studied in a wide variety of cell types. Ligand binding by the EGF receptor is associated with sequential tyrosine kinase activation of the receptor, increased activity of phospholipase C, hydrolysis of membrane lipids to yield diacylglycerols and inositol-triphosphate, increased calcium entry into cells, and activation of PKC with associated membrane translocation (50). In psoriatic epidermis, altered protein tyrosine kinase, phospholipase C, and PKC activities have been detected as well as elevated diacylglycerol levels (51-54). These changes could be due to chronic EGF receptor activation by TGF-α or to activation by other growth factors or pathways.

Despite extensive studies, the function of the EGF receptor system in normal and psoriatic human epidermis has not been precisely defined. One of the major limitations of these experiments is a lack of understanding of the growth factor requirements for normal human keratinocytes and the role of other growth factor pathways and their receptors in the regulation of keratinocyte proliferation. In fibroblasts, PDGF is a major mitogen "competence" factor which 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 (29,55). This thesis project was undertaken with the goal of further characterizing the function of each of the four principal tyrosine kinase-mediated growth factor pathways (EGF, IGF-I, FGF, and PDGF) in keratinocyte growth regulation. To address these issues, several different approaches were used combining both *in vitro* and *in vivo* studies of keratinocyte function.

The initial approach taken here to address growth regulation in keratinocytes was to determine individual and combined growth factor requirements of cultured normal human keratinocytes grown under standardized, totally defined culture conditions. These studies were designed not only to address the function of individual receptor systems but also to assess how interactions among these growth factor pathways might influence keratinocyte growth. These experiments then led to attempts to analyze alterations in growth factor receptor expression in response to a variety of culture conditions including treatment with different growth factors and differentiation inducing agents. Results obtained from these *in vitro* studies provided a rational basis for examining expression patterns of the major growth-related receptor systems in both normal and psoriatic skin.

While numerous treatments for psoriasis have been developed, the most commonly used ones were adopted purely on an empirical basis. Individual responses to treatment vary in an unpredictable manner and the mechanism by which any effective treatment modality works is not understood. A final goal of this work has been to begin studying the effects of anti-psoriatic agents on both the ligand and receptor components of growth factor pathways in cultured keratinocytes and on pre- and post-treatment psoriatic lesions. These studies might provide insights into the relative importance of different growth factor pathways in the psoriatic phenotype as well as advance our understanding of the mechanism of action of these specific therapies.

Chapter 2: The IGF-I Receptor in Cultured Human Keratinocytes and in Normal and Psoriatic Skin

2.1 Introduction

While the EGF receptor pathway is the best characterized keratinocyte growth factor pathway, the IGF-I receptor pathway might play a crucial role in the regulation of keratinocyte proliferation since IGF-I or high-dose insulin is necessary for the growth of human keratinocytes in standard serum-free culture systems (56). The work described in this chapter was undertaken with the goal of defining the role of the IGF-I receptor pathway in keratinocyte growth regulation through basic characterization of the pathway and its interactions with the EGF receptor system in regulating keratinocyte growth (57,58).

IGF-I, previously termed somatomedin C, is a 7.5 kd polypeptide that circulates in plasma in high concentrations and is detectable in most tissues (59). IGF-I, insulin, and IGF-II comprise a family of structurally related hormones that, nonetheless, produce distinct metabolic effects through interactions with unique cell surface receptors (60,61). IGF-I functions predominantly as a mitogenic factor for a variety of cell and tissue types, unlike insulin and IGF-II which serve predominantly as anabolic hormones, regulating glucose and mannose-6-phosphate intracellular transport, respectively (61). The importance of IGF-I to growth of post-embryonic tissues is suggested by increasing plasma concentrations throughout adolescence, reaching a plateau in adults, and in the requirement of most mammalian cell types for IGF-I for sustained proliferation (59).

The IGF-I receptor is composed of two subunits: α, a 125 kd protein which is entirely extracellular and functions in ligand binding, and β, a 95 kd transmembrane protein, with extracellular and cytoplasmic domains. The IGF-I receptor is synthesized as

a single chain propeptide which undergoes glycosylation, proteolytic cleavage, and assembly into a 350 kd heterodimer of both subunits ($\alpha_2\beta_2$) (62). The IGF-I receptor exhibits high affinity for binding of IGF-I, low affinity for binding of insulin, and intermediate affinity for binding of IGF-II (60). Thus, depending on ligand concentration, each of these factors has the potential to activate the IGF-I receptor after binding. Mitogenic signaling after ligand binding is produced by activation of tyrosine kinase enzymatic activity associated with the cytoplasmic portion of the IGF-I receptor β subunit. Although the insulin receptor possesses a structurally similar tyrosine kinase domain, the mitogenic properties of the IGF-I receptor are specific to unique sequences in its tyrosine kinase region (63). Like expression of the IGF-I ligand, tissue expression of the IGF-I receptor is also developmentally regulated (64).

The roles of IGF-I and its receptor in regulation of human skin structure and function are poorly understood. In connective tissue cells, such as fibroblasts, IGF-I regulates cell proliferation in conjunction with PDGF or other mitogens (29). However, keratinocyte growth regulation is likely to differ fundamentally from that of connective tissue cells since keratinocytes do not appear to express PDGF receptors ((23,30) and Chapter 4). In studies of mouse keratinocytes, IGF-I has been shown to regulate proliferation via synergistic interactions with EGF or FGF-like hormones (65). Sources of IGF-I in the epidermis have not been identified, but serum and dermal fibroblast production are likely sources. Recently, synthesis of IGF-I, possibly derived from keratinocytes or Langerhans cells, has been reported in ultraviolet irradiated skin (66). However, IGF-I synthesis by cultured human keratinocytes has not been detected. When this work was begun, little was known about IGF-I receptors in human skin beyond the description that epidermal keratinocytes (67,68) and a variety of dermal cell types such as fibroblasts and vascular elements express receptors for IGF-I (69,70).

2.2 Results

2.2.1 Keratinocyte Growth Requires EGF Combined with Either IGF-I or High-dose Insulin

Growth studies were initially attempted by seeding keratinocytes in complete serum free medium -- modified MCDB153 medium supplemented with hydrocortisone, EGF, insulin, and bovine pituitary extract (KGM) and switching the cells to unsupplemented medium (KBM) after 1 d. However, under these conditions keratinocytes continued to proliferate for at least 2 d consistent with previous reports (71) making the analysis of growth factor requirements difficult. Therefore, keratinocyte growth factor requirements were assessed by seeding keratinocytes in a minimal medium supplemented with growth factors individually or in combination, an approach used previously to define media requirements for clonal keratinocyte growth (32). Figure 2 shows the results of such a growth experiment in which KBM containing 0.5 mg/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. Figure 3 demonstrates the morphology of cells grown under these conditions at 1 and 6 d after seeding. 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 (Figure 3A and 3E). Cells seeded into medium containing EGF (Figure 3B and 3F) or high-dose insulin (Figure 3C and 3G) 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 (Figure 3F).

Figure 2 also demonstrates that a marked stimulation of keratinocyte proliferation occurs when either IGF-I or high-dose insulin is added in combination with EGF to the

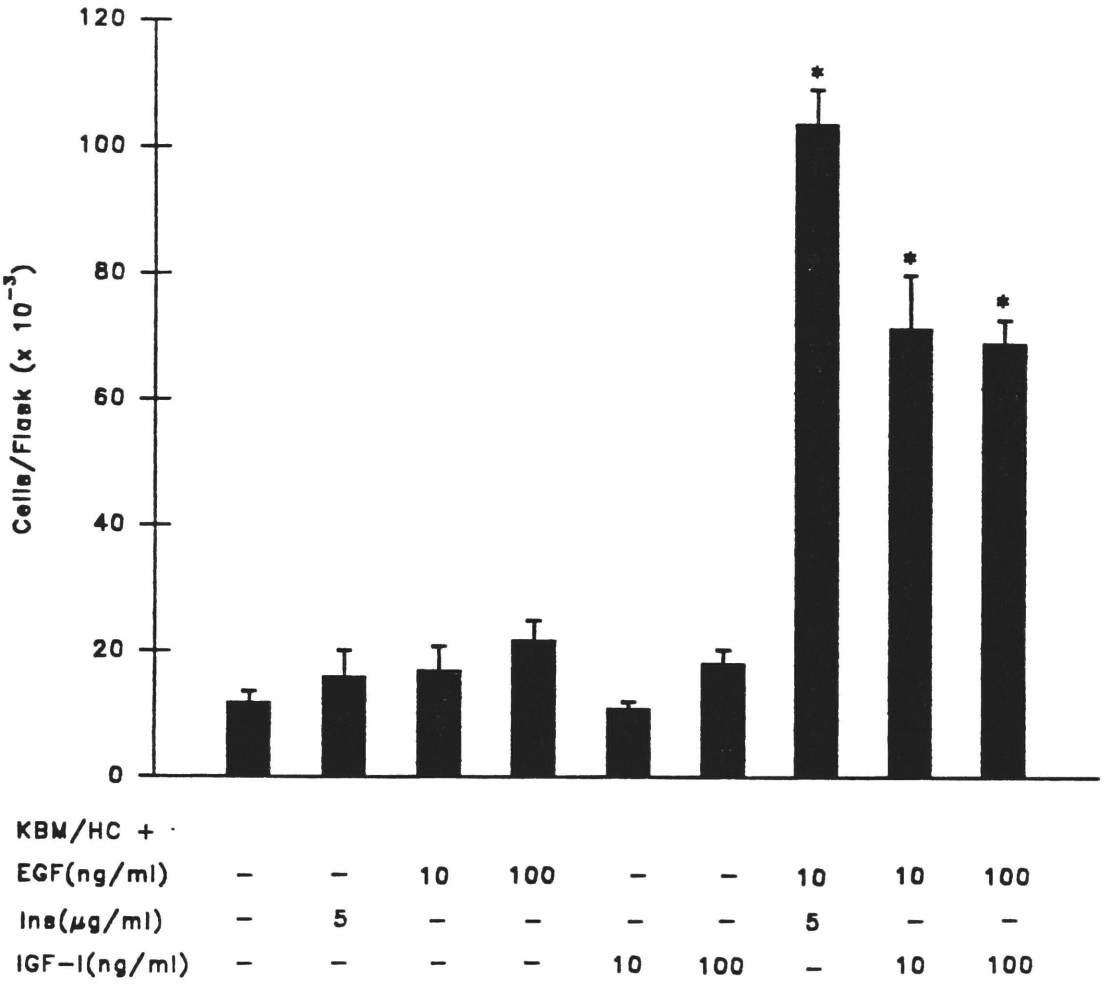
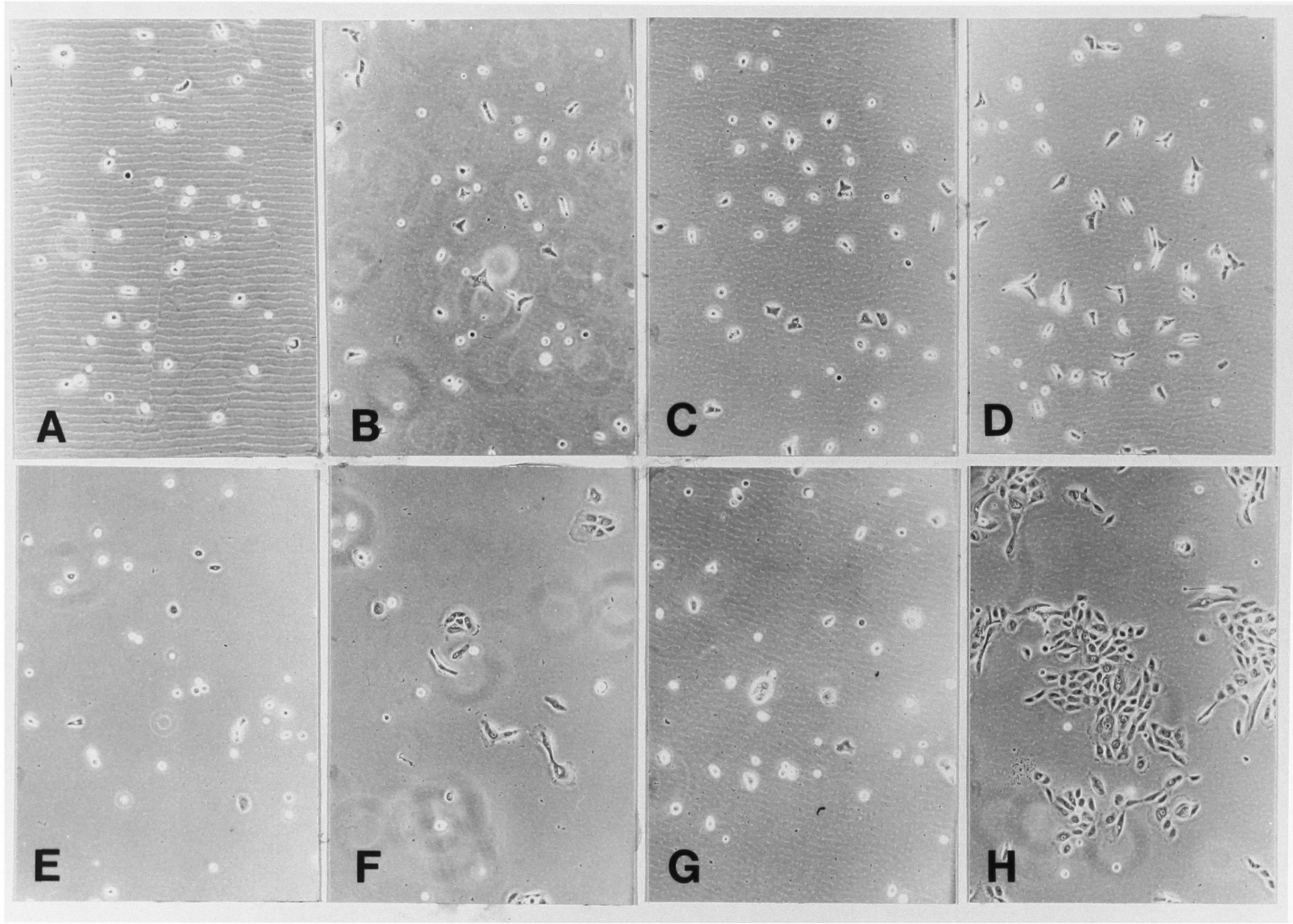


Figure 2. Synergistic interaction between IGF-I and EGF in promoting human keratinocyte growth. Cultured neonatal keratinocytes were seeded in KBM/HC supplemented with human recombinant factors as indicated. After 6 d, cells were trypsinized and counted in an electronic counter. Results represent the mean cell number of duplicate samples. The asterisk denotes a statistically significant ($P < 0.05$) difference compared to KBM/HC alone.

Figure 3. Keratinocyte morphology in the presence of different growth factors. Keratinocytes grown as described in Figure 2 legend were photographed 1 d (A-D) and 6 d (E-H) after seeding in KBM/HC alone (A,E) or supplemented with 10 ng/ml human recombinant EGF (B,F), 5 μg/ml human recombinant insulin (C,G), or both EGF and insulin (D,H). Note that some spreading of keratinocytes can be seen in KBM/HC + EGF at both 1 d (B) and 6 d (F). However, only in the presence of both EGF and insulin together does significant proliferation occur (H). Similar results were obtained when IGF-I was substituted for insulin or when KBM was substituted for KBM/HC (not shown).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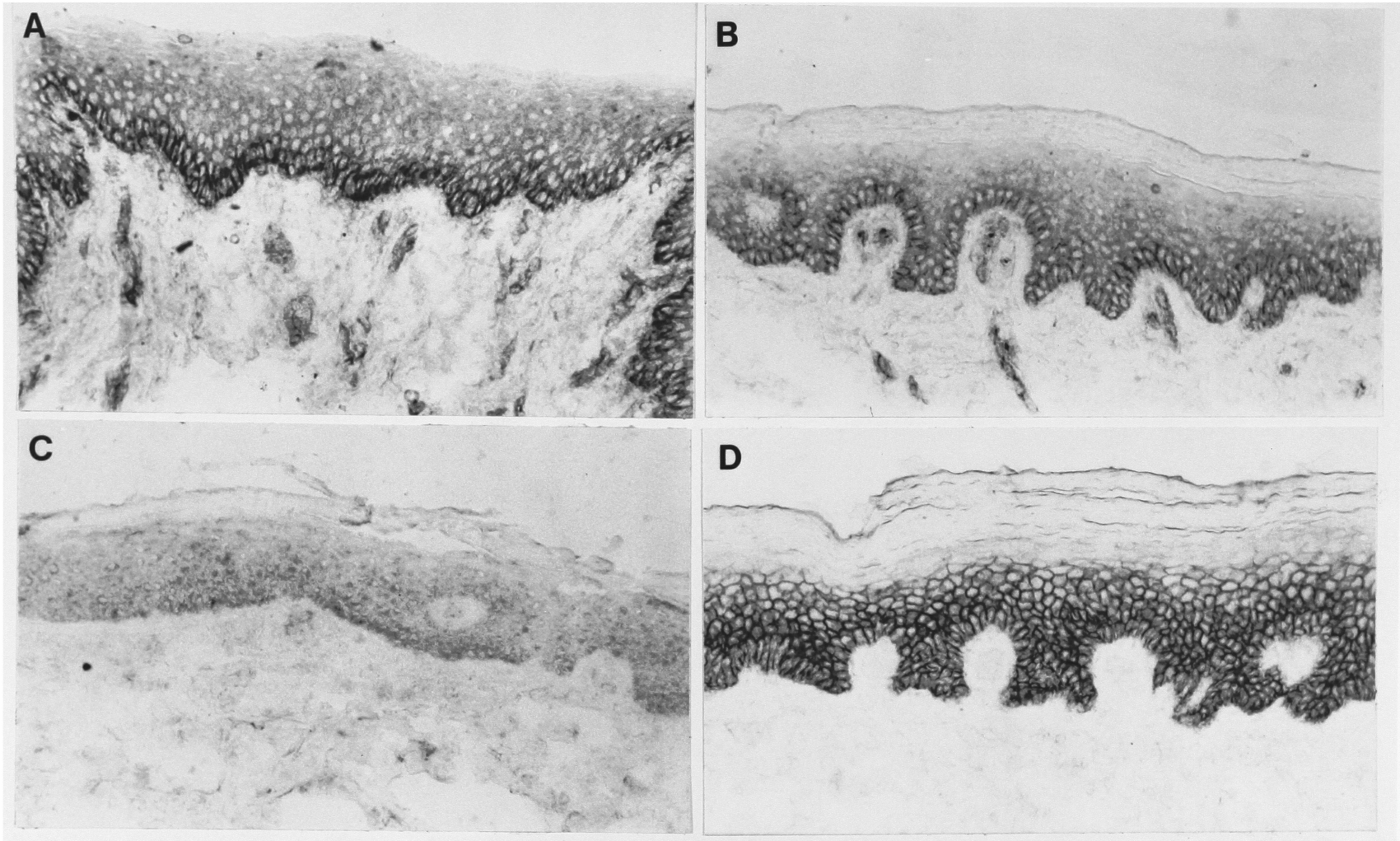
basal growth medium. In Figure 3D and 3H, a considerably enhanced degree of cell spreading is evident by 1 d after seeding with high-dose insulin and EGF. After an additional 5 d, typical colonies of proliferating keratinocytes are evident. Thus the results in Figures 2 and 3 indicate that activation of the EGF and IGF-I receptor systems together, but not either system individually, is sufficient to promote keratinocyte spreading and proliferation. This experiment suggested that the IGF-I receptor pathway might play an important role in regulating epidermal growth which warranted further study of IGF-I receptor expression *in vivo* and *in vitro*.

2.2.2 IGF-I Receptors are Expressed on the Surface of Basal Keratinocytes in Normal Human Epidermis

If the coactivation of both EGF and IGF-I receptor pathways observed in cultured keratinocytes has any relevance to *in vivo* growth regulation, one would predict that keratinocytes in the basal proliferative compartment of the epidermis should coexpress both EGF and IGF-I receptors. Previous immunohistochemical studies (72) have already established that EGF receptors are expressed throughout all viable epidermal layers. Although the presence of IGF-I receptors on the surface of cultured human keratinocytes has been previously reported (67,68), the *in vivo* pattern of IGF-I receptor expression has not been described. Therefore, immunohistochemical analysis of IGF-I receptor expression was performed on samples of normal human skin. In Figure 4A and 4B, 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, αIR-3 (73). IGF-I receptors are expressed on the cell surface of keratinocytes in the basal, proliferative cell compartment of normal human epidermis.

Figure 4. 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 using antibodies directed against the IGF-I receptor (A,B), the EGF receptor (D), and a non-specific isotype control (C). Note that IGF-I receptor membrane staining is confined to the basal proliferative cell layer of the epidermis (A,B). In contrast, EGF receptor membrane staining is seen in all viable layers of the epidermis (D).

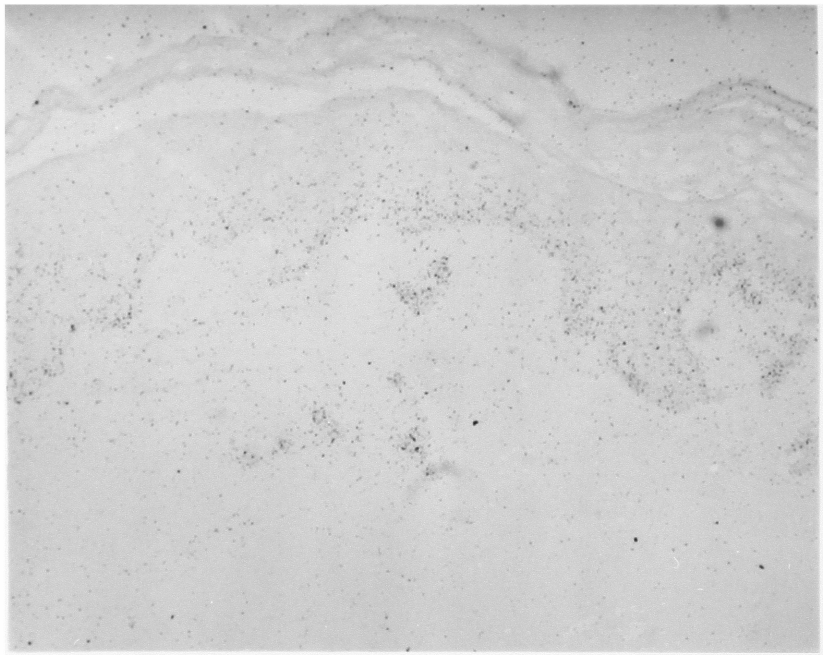

A
B
C
D

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 (Figure 4C). An EGF receptor monoclonal antibody used at the same antibody concentration stained all viable epidermal layers (Figure 4D) in agreement with its previous description in normal human epidermis (72). 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. In contrast to the pattern of EGF receptor expression, IGF-I receptor distribution correlates strictly with the proliferative compartment of the epidermis in normal epidermis.

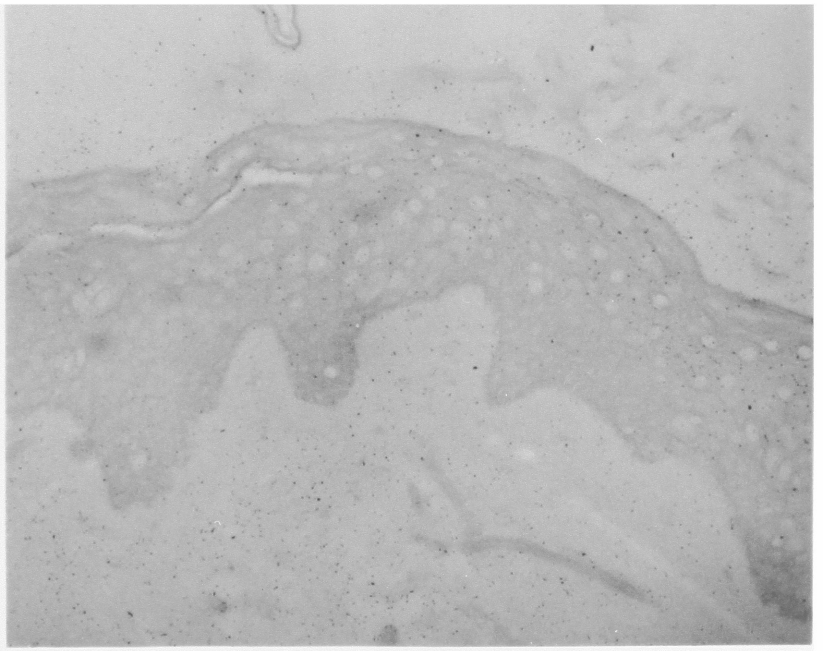
While EGF receptors can be identified throughout the viable layers of the epidermis by immunohistochemistry, ^{125}I-EGF binding in normal human skin is confined primarily to the basal cell layer (48). To test whether IGF-I receptor ligand binding and immunohistochemical staining correlate with each other, binding of ^{125}I-IGF-I to normal human skin was measured as shown in Figure 5. ^{125}I-IGF-I binding localizes to the basal cell region of the epidermis in agreement with the staining data presented in Figure 4. Incubation in the presence of excess unlabelled ligand effectively competes for radioligand binding. It must be noted that the ^{125}I-IGF-I binding observed in this experiment could also reflect binding of the radioligand to insulin or IGF-II receptors in the epidermis, but the experiment is consistent with the immunologically identified IGF-I receptor distribution coinciding with IGF-I ligand binding sites.

Figure 5. Distribution of ^{125}I-IGF-I binding in normal adult human skin. In the section on the left, tissue was incubated with 5 ng/ml ^{125}I-IGF-I alone, while in the section on the right a 5,000-fold excess of unlabelled IGF-I was added along with the radioligand. Silver grains are distributed predominantly over the basal cell layer of the epidermis (and over dermal blood vessels) in the presence of radioligand alone, while no specific distribution of silver grains is detectable in the presence of excess IGF-I.

^{125}I-IGF-1



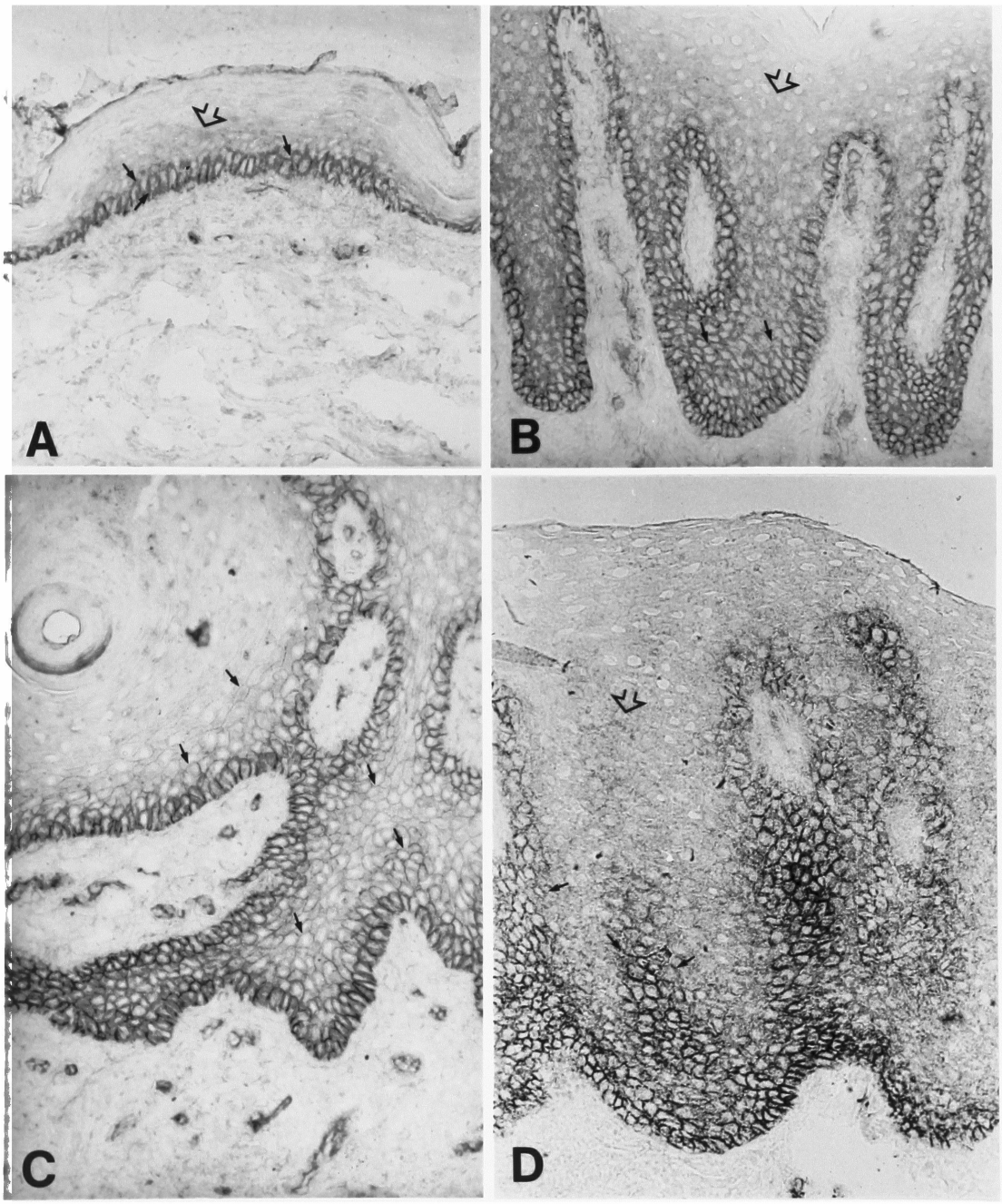
^{125}I-IGF-1 + excess IGF-1



2.2.3 Analysis of IGF-I Receptor Expression in Psoriatic Epidermis by Immunohistochemistry

The expression of the IGF-I receptor may define the potential proliferative pool of keratinocytes in epidermal tissue. As the proliferative keratinocyte population extends to suprabasal keratinocytes in lesional psoriatic epidermis (6), the expression of IGF-I receptors was compared in lesional and non-lesional psoriatic epidermis by immunohistochemistry with the αIR-3 antibody. Biopsy samples of active psoriatic plaques and normal appearing non-lesional skin from 18 patients were sectioned on a cryostat microtome and processed for immunoperoxidase staining using αIR-3 antibody. Representative results from this analysis are shown in Figure 6. In contrast to normal human epidermis, acanthotic epidermis from lesional psoriatic plaques shows plasma membrane staining with αIR-3 in both basal keratinocytes and in lower spinous keratinocytes (Figure 6B-D), corresponding to the increased proliferative keratinocyte compartment in lesional psoriatic epidermis (6). Biopsy tissue from 18 individuals with active psoriatic lesions consistently showed this pattern of IGF-I receptor staining at cell surfaces of basal and lower spinous epidermal layers. Diffuse, cytoplasmic staining was variably seen in mid and upper spinous keratinocyte layers. IGF-I receptor expression was also studied in normal appearing, non-lesional skin from 15 of these patients with active psoriatic lesions (Figure 6A). Cell surface IGF-I receptor expression in 13/15 tissue samples (87%) was confined to the basal epidermal layer, as described in skin from normal individuals (Figure 4). Diffuse, cytoplasmic staining of IGF-I receptors was also seen in some suprabasal keratinocytes. Two other samples of non-lesional skin from psoriatics showed focal areas of suprabasal membrane staining for the IGF-I receptor along with basal staining.

Figure 6. Immunohistochemical reactivity of psoriatic epidermis with the anti-IGF-I receptor mAb, αIR-3. Intense membrane staining (arrows) of both basal and suprabasal keratinocytes is observed in psoriatic lesional epidermis (B-D), while plasma membrane reactivity is confined to the basal layer of keratinocytes in normal appearing, non-lesional skin (A). Weaker cytoplasmic staining of spinous keratinocytes with the mAb is also detected as indicated by the open arrowheads.


A
B
C
D

2.2.4 Monoclonal Antibody αIR-3 Recognizes the IGF-I Receptor in Cultured Human Keratinocytes

The monoclonal antibody αIR-3 was raised to IGF-I receptors purified from human placental membranes (73). AlphaIR-3 reacts with an antigenic site on the extracellular α-subunit of the IGF-I receptor (73) and its specificity has been demonstrated in a variety of cell types (73-76). To further substantiate the ability of αIR-3 to specifically recognize the IGF-I receptor in human keratinocytes, radioreceptor competition and immunoprecipitation experiments were performed with cultured normal human keratinocytes. Figure 7 displays results obtained with radioreceptor competition experiments. In this experiment, the ability of IGF-I, insulin, and αIR-3 to compete binding of ^{125}I-IGF-I was measured on keratinocyte monolayers. AlphaIR-3 displayed an ability to inhibit binding of ^{125}I-IGF-I to its receptor to nearly the same extent as IGF-I, competing for radioligand to a greater degree than did a 10,000-fold excess concentration of insulin.

The specificity of αIR-3 binding to the IGF-I receptor was also confirmed by immunoprecipitation of the receptor from keratinocyte lysates followed by autophosphorylation in an *in vitro* kinase reaction (Figure 8). The 95 kd β-subunit of the IGF-I receptor was detected only when cells were briefly exposed to IGF-I prior to immunoprecipitation (Figure 8, lanes B,C).

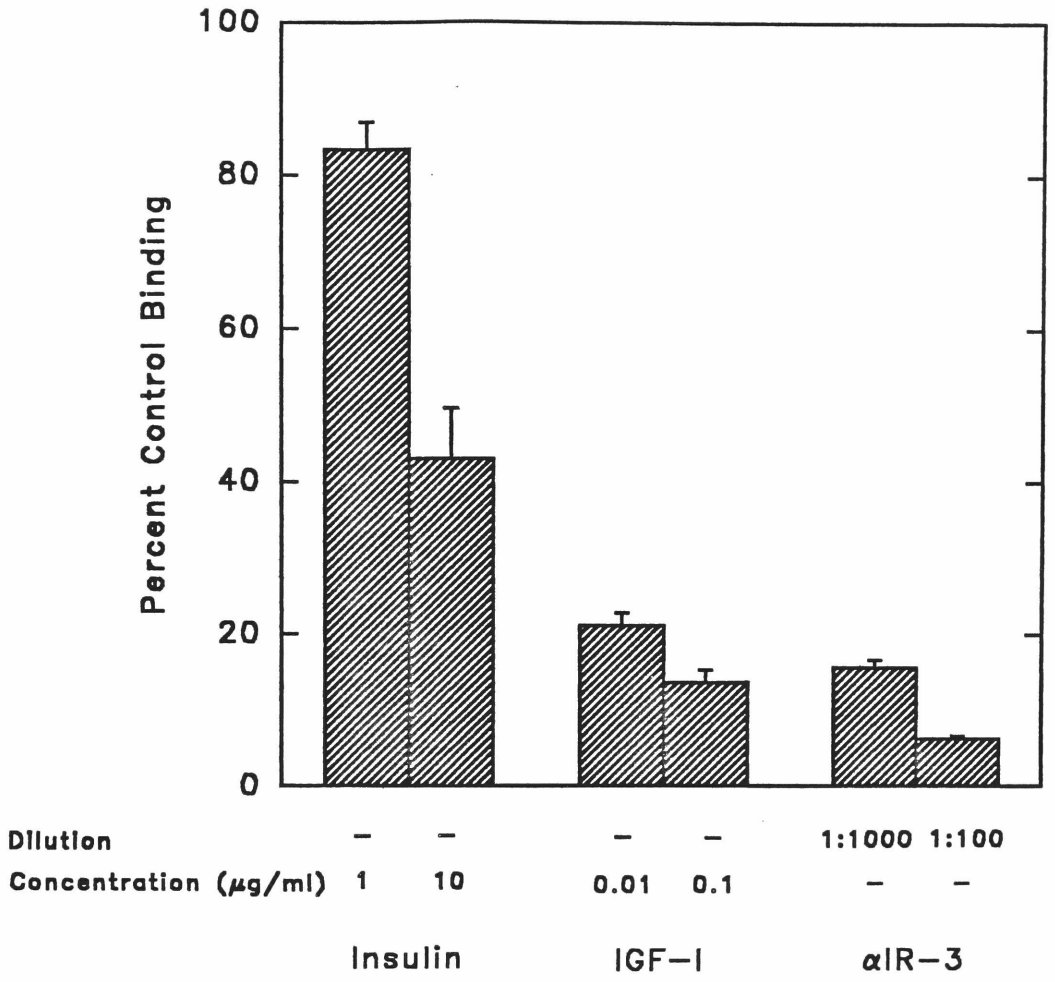
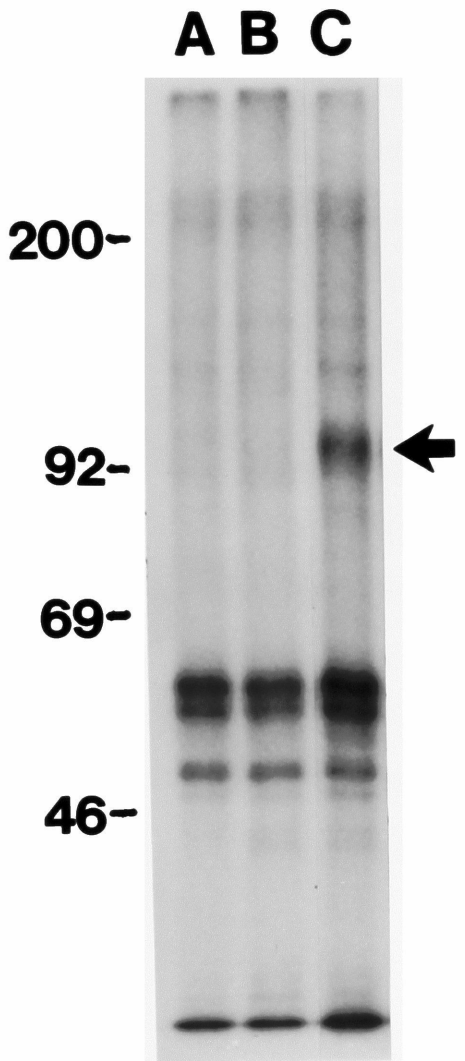


Figure 7. Monoclonal antibody αIR-3 inhibits binding of ^{125}I-IGF-I to cultured human keratinocytes. ^{125}I-IGF-I (1 ng/ml) binding was measured in the presence of the indicated concentrations of bovine insulin (Clonetics Corp.), human recombinant IGF-I (Pepro Tech Corp.) , and αIR-3. Keratinocytes grown in KGM were transferred to KBM for 1 d prior to the assay. Data are expressed as the percentage of counts bound in the condition tested/counts bound in KBM alone.

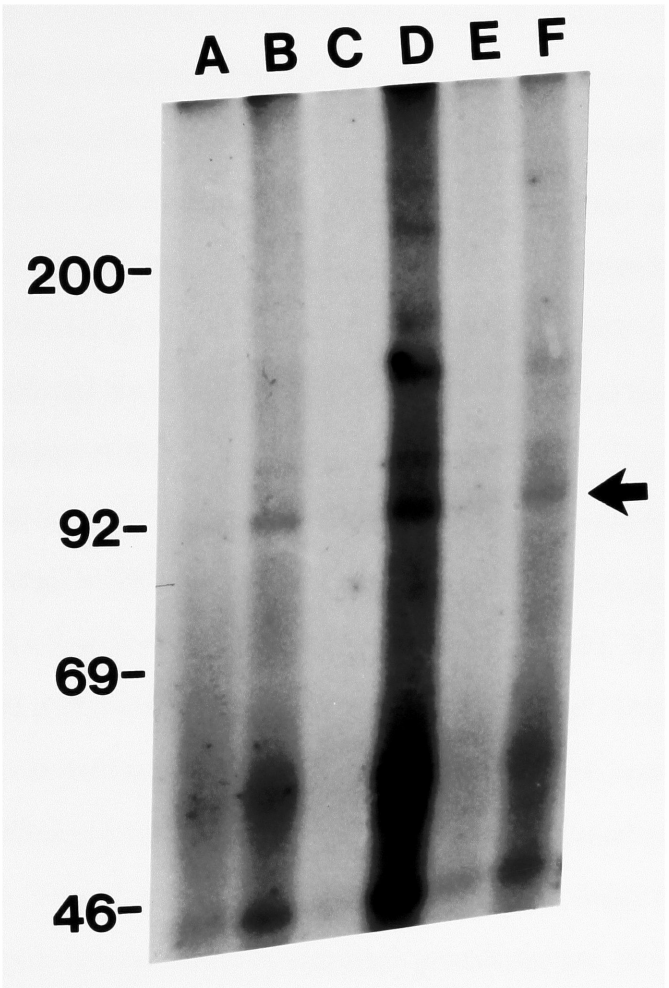
Figure 8. Monoclonal antibody αIR-3 immunoprecipitates the IGF-I receptor in cultured human keratinocytes. Cells were grown in basal medium for 24 h prior to lysis and the sample in lane C was treated with 100 ng/ml IGF-I for the last 30 min. Cell lysates were immunoprecipitated with αIR-3 (lanes B and C) or an isotypic control antibody (lane A) and then autophosphorylated by an *in vitro* kinase assay in the presence of γ-^{32}P-ATP. The arrow indicates the 95 kd β subunit of the IGF-I receptor. IGF-I receptor autophosphorylation was only detected when the receptor kinase was activated by addition of IGF-I (compare B to C). Non-specific bands in the 46-69 kd size range are probably phosphorylated keratins.


A B C
200-
92-
69-
46-

2.2.5 Analysis of IGF-I Receptor Expression in Psoriatic Epidermis by Immunoprecipitation and Kinase Assay

To confirm the apparent increase in IGF-I receptor expression in psoriatic epidermis, IGF-I receptor kinase activity was assessed in psoriatic tissue. Split-thickness biopsies consisting predominantly of epidermal tissue were obtained from lesional and non-lesional skin from 3 different individuals. Equal quantities of homogenized tissue protein were immunoprecipitated with αIR-3 and then receptor autophosphorylation was initiated by the addition of γ-^{32}P-ATP. Kinase reaction samples were separated by SDS-PAGE with the results shown in Figure 9. In all 3 paired samples, IGF-I receptor kinase activity as measured by receptor autophosphorylation was greater in psoriatic tissue than in the paired non-lesional specimen. Densitometry measurements showed a 5 to 20-fold increase in kinase activity in psoriatic epidermis compared with paired nonlesional specimens. In contrast with the kinase assay on cultured keratinocytes in Figure 8, IGF-I receptor kinase activity is detectable in psoriatic tissue without the addition of exogenous IGF-I (despite the use of approximately 5-fold less total protein from the biopsy samples than with the cultured keratinocytes). The increased kinase activity of IGF-I receptors is consistent with the immunohistochemical finding of increased IGF-I receptor expression in psoriatic epidermis and further indicates that these receptors are both biologically functional and activated *in vivo*.

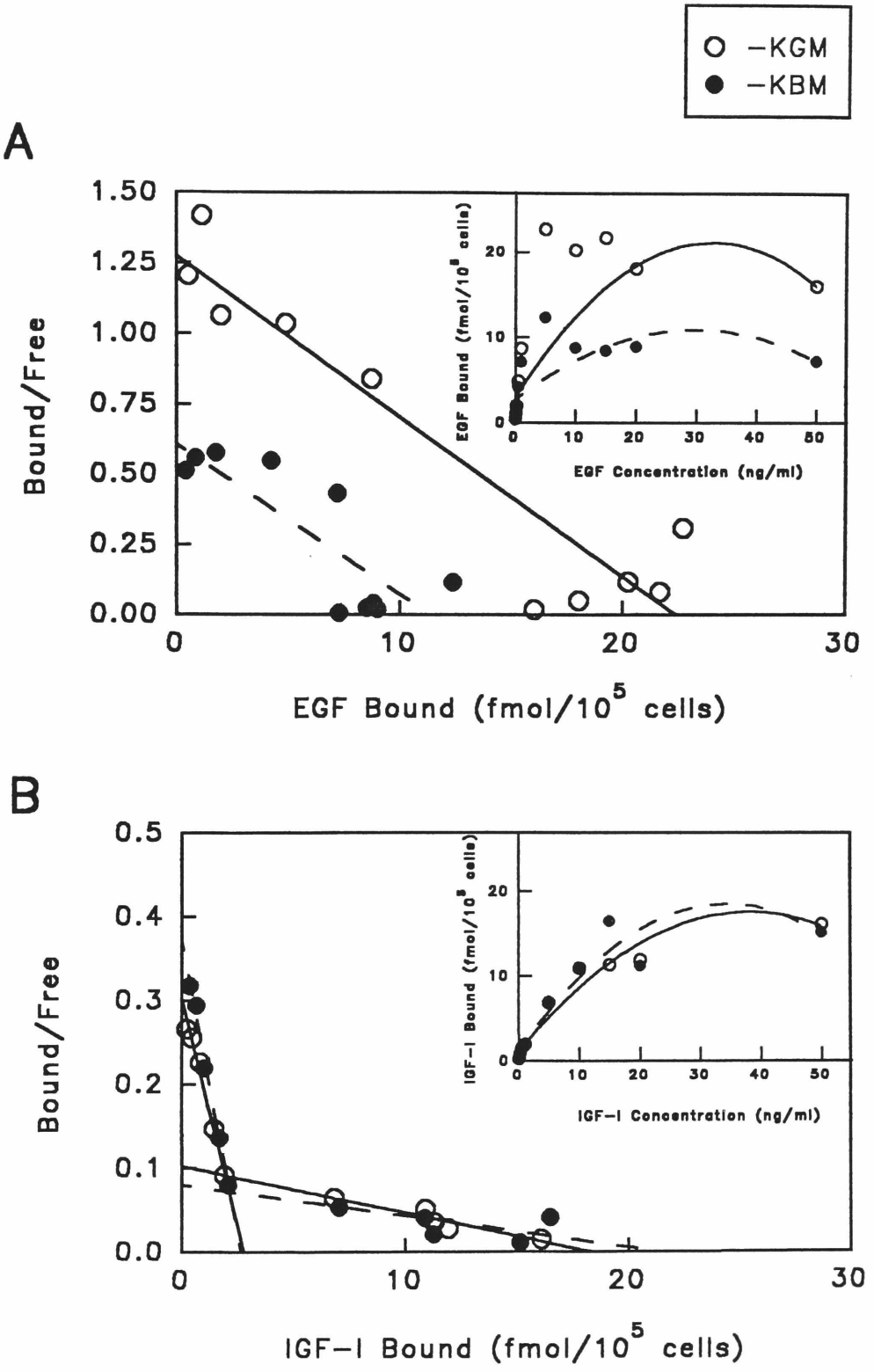
Figure 9. IGF-I receptor kinase activity is increased in psoriatic epidermis. Sixty μg of protein from non-lesional (lanes A,C,E) and lesional (lanes B,D,F) split-thickness biopsies of patients 1 (lanes A and B), 2 (lanes C and D), and 3 (lanes E and F) were immunoprecipitated with mAb αIR-3 and then autophosphorylated with γ-^{32}P-ATP. The arrow indicates the 95 kd β subunit of the IGF-I receptor.


A B C D E F
200-
92-
69-
46-

2.2.6 Differential Modulation of IGF-I Receptor and EGF Receptor Expression in Human Keratinocytes

Even with the apparent increase in IGF-I receptor expression in lesional psoriatic epidermis, it should be noted that the immunohistochemical findings indicate that there is much more restricted expression of the IGF-I receptor in normal or psoriatic skin compared to expression of the EGF receptor, which is present throughout the viable epidermal layers of psoriatic epidermis (48,72). This suggested that expression of the IGF-I receptor and the EGF receptor might be regulated by different biochemical pathways in keratinocytes or in epidermal tissue. Expression of IGF-I and EGF receptors was examined in cultured human keratinocytes to analyze potential similarities or differences in regulation of these growth factor receptor systems. Figure 10 shows binding of ^{125}I-IGF-I and ^{125}I-EGF over a range of ligand concentrations to keratinocyte monolayers maintained in KBM with and without added exogenous growth factors. This figure also displays a Scatchard transformation of the binding data. Keratinocytes maintained in KGM expressed approximately twice as many EGF receptors as cells maintained for 24 h in unsupplemented basal medium. In contrast, expression of the IGF-I receptor was not affected by growth factor supplementation of basal medium over a 24 h period (Figure 10). These data suggest that the IGF-I receptor might be less sensitive than the EGF receptor to modulation by unrelated growth factors. Note that the binding curve for IGF-I can be resolved into high and low affinity receptor components. The high affinity binding certainly represents binding of IGF-I to its receptor, but it is possible that all or part of the low affinity component could be binding of ^{125}I-IGF-I to insulin or IGF-II receptors. Based solely on the high affinity receptor component, it appears that human keratinocytes have fewer IGF-I receptors than EGF receptors under identical culture conditions. If low affinity receptor sites for IGF-I are considered, then keratinocytes

Figure 10. Growth factor supplementation increases EGF receptor binding sites on cultured human keratinocytes, but does not affect IGF-I receptor binding. Scatchard analysis was performed with ^{125}I-EGF (A) and ^{125}I-IGF-I (B) for keratinocytes grown in KGM and then transferred to KBM (closed circles) or KGM (open circles) for 24 h prior to the assay. The Scatchard plot in (A) indicates that EGF receptor binding sites are increased in KGM compared to KBM with no change in binding affinity observed. The plot in (B) demonstrates that both high and low affinity IGF-I binding sites are unchanged by growth in KGM. The inserts in each panel are plots of the concentration of ligand added versus specific binding of the ligand.

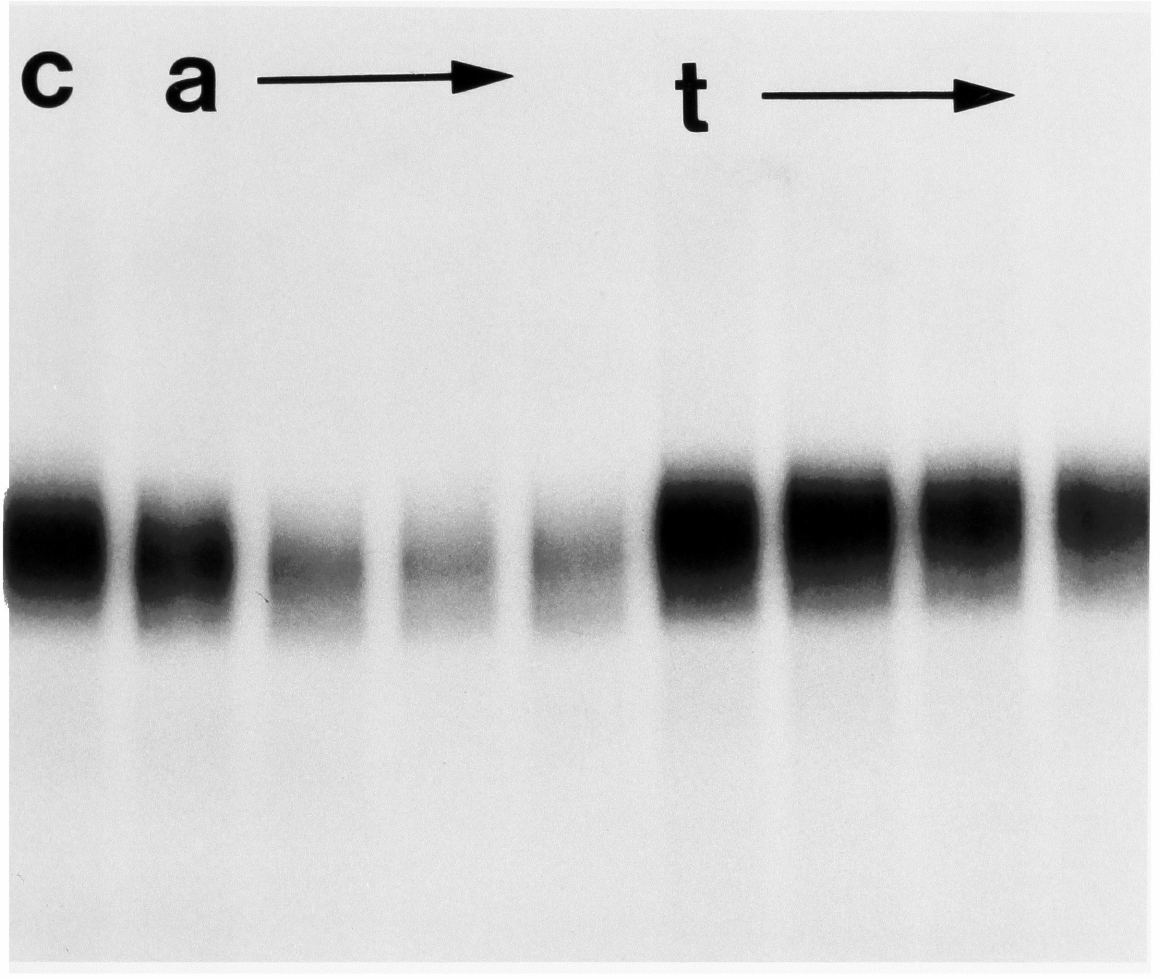

○ −KGM
● −KBM
A
Bound/Free
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
10
20
30
EGF Bound (fmol/10^5 cells)
EGF Bound (fmol/10^5 cells)
EGF Concentration (ng/ml)
B
Bound/Free
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
IGF−I Bound (fmol/10^5 cells)
IGF−I Bound (fmol/10^5 cells)
IGF−I Concentration (ng/ml)

grown in KGM would express approximately equal numbers of IGF-I and EGF receptors.

A number of pharmacological agents have significant effects on binding of EGF to its receptor, either through effects on cell surface receptor number or through an altered binding affinity of the receptor for EGF. Activation of PKC by phorbol esters or calcium-ionophore A23187 in keratinocyte cell lines results in phosphorylation of EGF receptors on threonine residue 654, causing a rapid decrease in EGF receptor binding affinity and in EGF receptor tyrosine kinase activity (77-80). Figure 11 shows a time course kinase reaction demonstrating that even short-term treatment of normal cultured human keratinocytes with either PMA or ionophore A23187 and high calcium concentrations results in a rapid decrease in EGF receptor autophosphorylation evident within a period of less than 20 min. Control samples treated with 4α-PMA, an inactive isomer of PMA, showed no alteration of kinase activity (data not shown).

Given differences in the tissue distribution of IGF-I and EGF receptors in human epidermis, we sought to compare effects of these potent pharmacological agents on expression of IGF-I and EGF receptors. In the experiment shown in Figure 12, binding of ^{125}I-IGF-I or ^{125}I-EGF to keratinocyte monolayers was determined after treatment with PMA for times up to 22 h. As a control for non-specific effects of phorbol compounds that are unrelated to PKC activation, binding has been compared to treatment with 4α-PMA. As expected, treatment of keratinocytes with PMA produced a rapid decrease in ^{125}I-EGF binding, which was maximally depressed at 22 h treatment. In contrast, the opposite effect on binding of ^{125}I-IGF-I was produced by PMA treatment, with increased binding of ^{125}I-IGF-I evident by 8 h treatment. Treatment of keratinocytes over this period with 4α-PMA produced no significant change in ^{125}I-EGF or ^{125}I-IGF-I binding. To determine whether these changes in ligand binding were due to alterations of receptor number or binding affinity, binding of these ligands to PMA treated cells was examined over a range of ligand concentrations and binding data were transformed to a Scatchard plot. PMA treatment decreased EGF receptor binding affinity from Kd≈0.4 nM to Kd≈10

Figure 11. EGF receptor kinase autophosphorylation decreases in response to PMA or ionophore A23187 treatment of cultured human keratinocytes. Keratinocytes grown in KGM were treated with DMSO alone as a control (c), 1.15 mM $CaCl_2$/ 10 μM A23187 (a), or 100 ng/ml PMA (t). Following the direction of the arrows, keratinocytes were treated with ionophore or PMA for 5, 10, 15, or 20 min prior to lysis. Cell lysates were immunoprecipitated with a rabbit anti-EGF receptor antibody and then autophosphorylated by an *in vitro* kinase assay in the presence of γ-^{32}P-ATP.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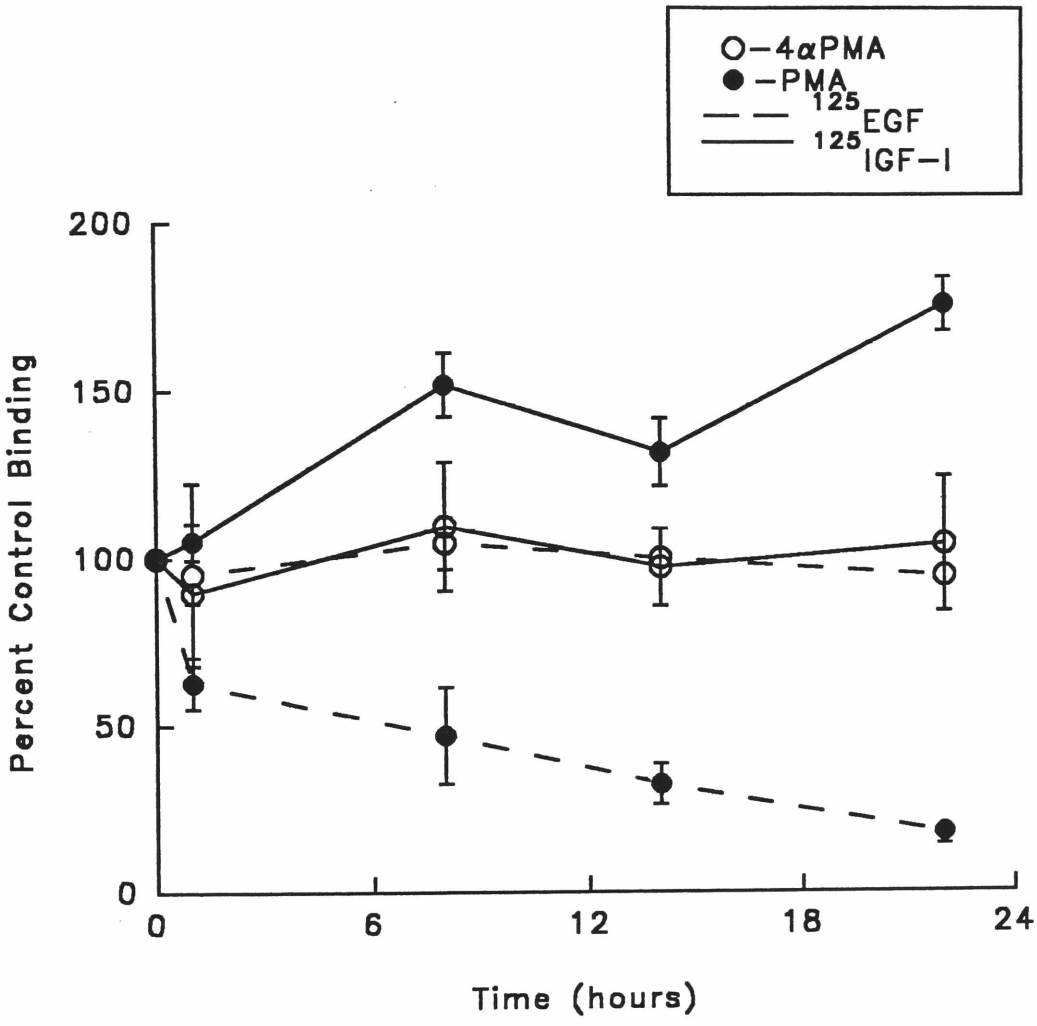


Figure 12. PMA treatment of human keratinocyte monolayers has opposite effects on ^{125}I-EGF and ^{125}I-IGF-I binding. Keratinocytes in KBM were treated for 0-22 h with PMA (closed circles) or the inactive phorbol analog 4α-PMA (open circles). Solid lines indicate ^{125}I-IGF-I binding while the dashed lines denote ^{125}I-EGF binding. Control binding was performed on cells treated with KBM containing solvent alone.

nM with no apparent change in receptor number (Figure 13). In contrast, PMA treatment increased the affinity of high affinity ^{125}I-IGF-I binding sites from Kd≈2 nM to Kd≈0.6 nM (Figure 14) with no significant alteration of low affinity ^{125}I-IGF-I binding sites (not shown).

Since PKC, as well as a number of other enzymes, can be activated by increased intracellular calcium ions, keratinocytes were treated with increased extracellular calcium (1.15 mM) or ionophore A23187 with and without increased extracellular calcium. The effect of these treatments was then measured on binding of ^{125}I-IGF-I and ^{125}I-EGF to keratinocyte monolayers (Figure 15). Treatment of keratinocytes with increased extracellular calcium and with A23187 produced a time dependent decrease in EGF binding, though the overall effect was not as large as that produced by PMA treatment. In contrast, a time dependent increase in ^{125}I-IGF-I binding was produced by all treatments, though A23187 with increased extracellular calcium produced the largest increase. Scatchard analysis of the A23187 effect on EGF and IGF-I binding (Figures 13 and 14), showed that the decreased EGF binding was largely a function of reduced receptor number, whereas the increased IGF-I binding was produced by an increased affinity of high affinity binding sites. Increased affinity of high affinity IGF-I receptors was also produced by treatment of keratinocyte monolayers with ionomycin in the presence of increased (1.15 mM) extracellular calcium. Ionomycin is an ionophore with higher selectivity for intracellular calcium transport than A23187 (81), suggesting that the effect of ionophore A23187 on IGF-I receptor expression is mediated through increased calcium transport and not by another divalent cation.

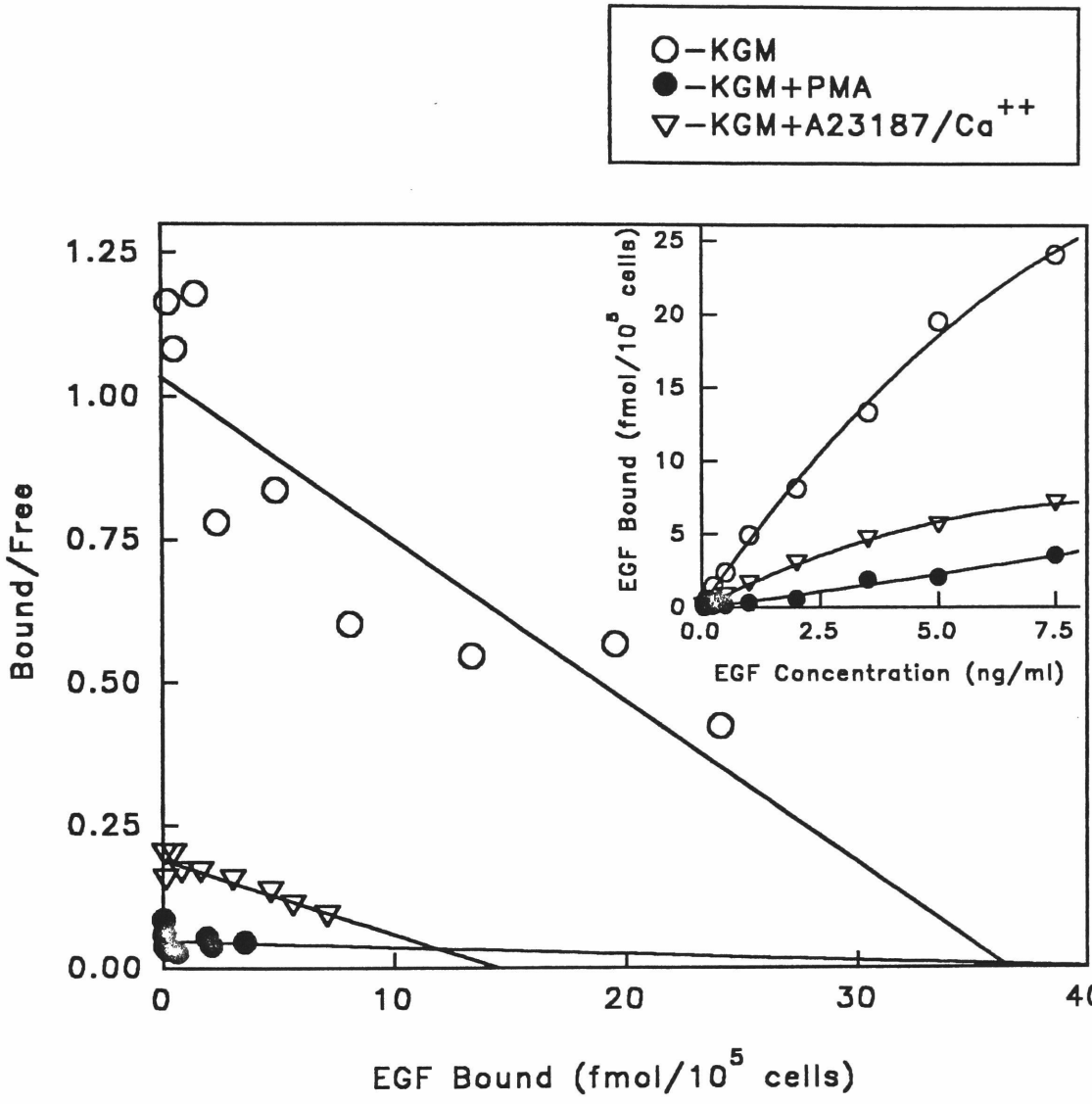


Figure 13. Scatchard analysis of the effects of PMA and Ca^{++}/A23187 on EGF receptor binding to cultured human keratinocytes. Keratinocytes were treated for 24 h with KGM alone (open circles) or containing 10 ng/ml PMA (closed circles) or 1.15 mM $CaCl_2$/0.1 μM A23187 (open triangles). The insert is a plot of EGF concentration added versus specific binding of the ligand.

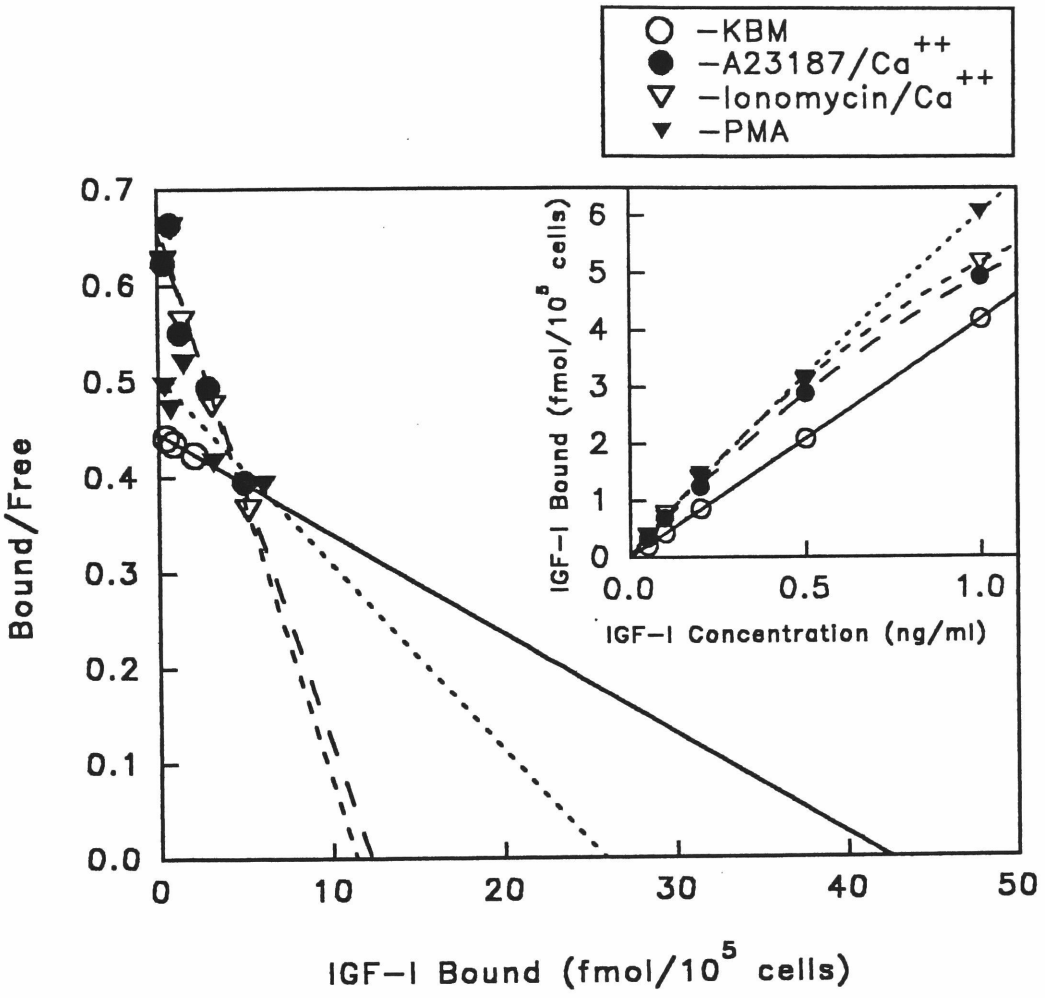


Figure 14. Scatchard analysis of the effects of PMA, Ca^{++}/A23187, and Ca^{++}/ionomycin on high affinity IGF-I receptor binding to cultured human keratinocytes. Keratinocytes were treated for 24 h with KBM alone (open circles, solid line) or containing 10 ng/ml PMA (closed triangles, dotted line) or 1.15 mM $CaCl_2$ with either 0.1 μM A23187 (closed circles, long-dashed line) or 1 μM ionomycin (open triangles, short-dashed line). The insert is a plot of IGF-I concentration added versus specific binding of the ligand. Low affinity IGF-I binding has been omitted from the figure for the sake of clarity.

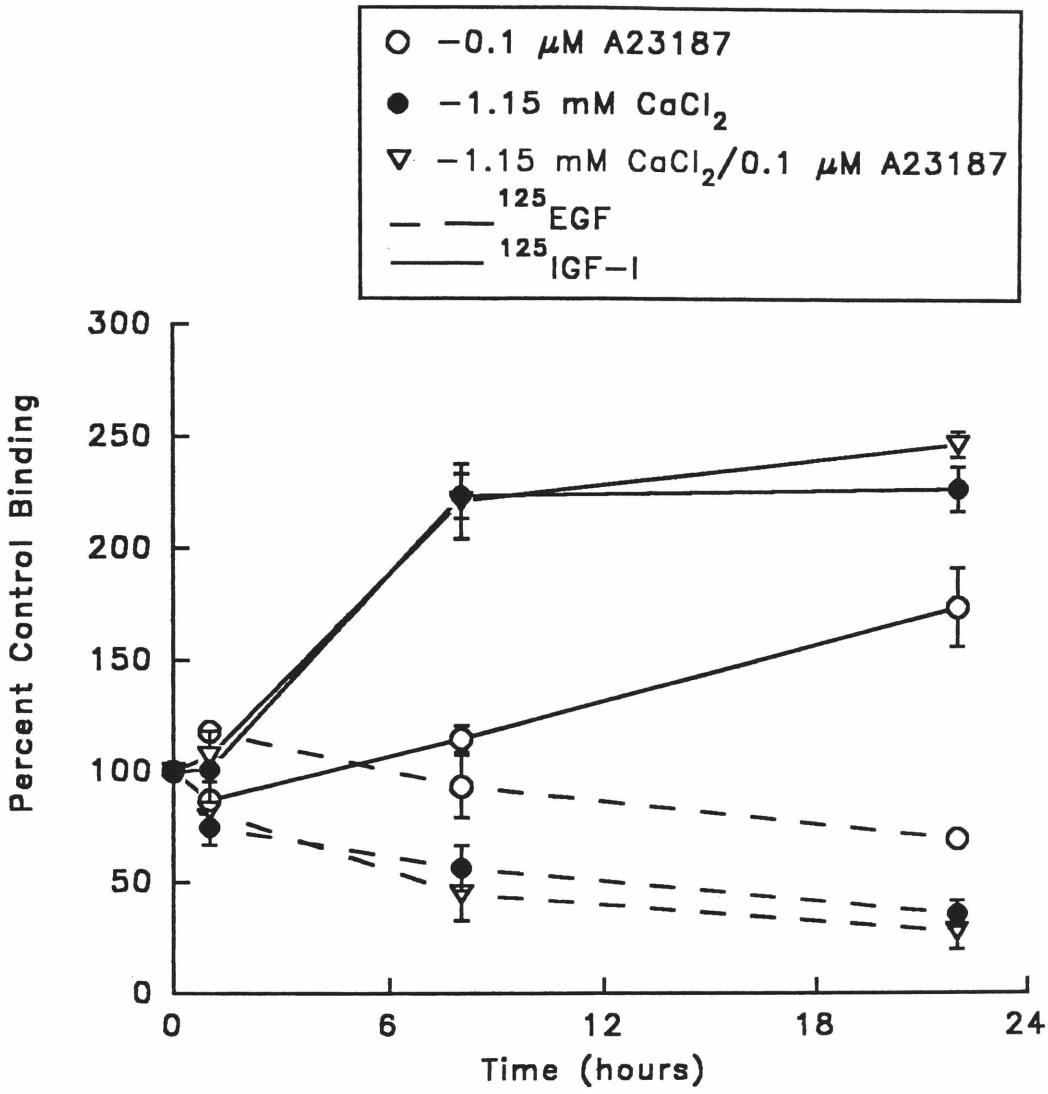


Figure 15. Calcium and/or calcium ionophore treatment of keratinocyte monolayers has opposite effects on ^{125}I-EGF and ^{125}I-IGF-I binding. Keratinocytes in KBM were treated for 0-22 h with 1.15 mM $CaCl_2$ (closed circles), 0.1 μM A23187 (open circles), or both (open triangles). Solid lines indicate ^{125}I-IGF-I binding while the dashed lines denote ^{125}I-EGF binding. Control binding was performed on cells treated with KBM containing solvent alone.

2.2.7 IGF-I or High-dose Insulin Increases EGF Binding to Keratinocytes

The experiment described in Figure 10 indicates that one or more of the supplemented growth factors in KGM -- IGF-I, EGF, hydrocortisone, or bovine pituitary extract contributes to increasing cell surface EGF receptor numbers. Since the growth experiment described in Figure 2 indicates that the EGF and IGF-I receptor pathways act synergistically, experiments were performed to test whether alteration of EGF receptor binding properties by IGF-I treatment of keratinocytes might occur. Such "transmodulation" (82), in which activation of one receptor pathway regulates another receptor pathway by directly or indirectly altering receptor affinity, receptor number, and/or receptor tyrosine kinase activity, could potentially contribute to the growth synergy between the IGF-I and EGF receptor systems.

In the growth assay described in Figure 2, insulin and IGF-I were used interchangeably since previous work has indicated that the keratinocyte growth requirement for supraphysiologic insulin concentrations can be replaced by IGF-I (83) and is mediated through low affinity insulin binding to and activation of the IGF-I receptor (67,68,84). The competition experiment shown in Figure 16 verifies that insulin does compete for IGF-I receptor binding in cultured human keratinocytes, but only at high doses of insulin. A 50-fold excess of unlabelled IGF-I competed greater than 90% of radioligand binding whereas a 10,000-fold excess insulin concentration only displaced 57% binding of IGF-I. Considerable displacement of IGF-I binding occurs only at the supraphysiologic insulin concentrations typically used in serum-free culture systems (56,83,84). In the remaining experiments described in this thesis, IGF-I and high-dose insulin had essentially the same effects on growth of cultured keratinocytes consistent with their mitogenic activity being mediated exclusively via activation of the IGF-I receptor pathway.

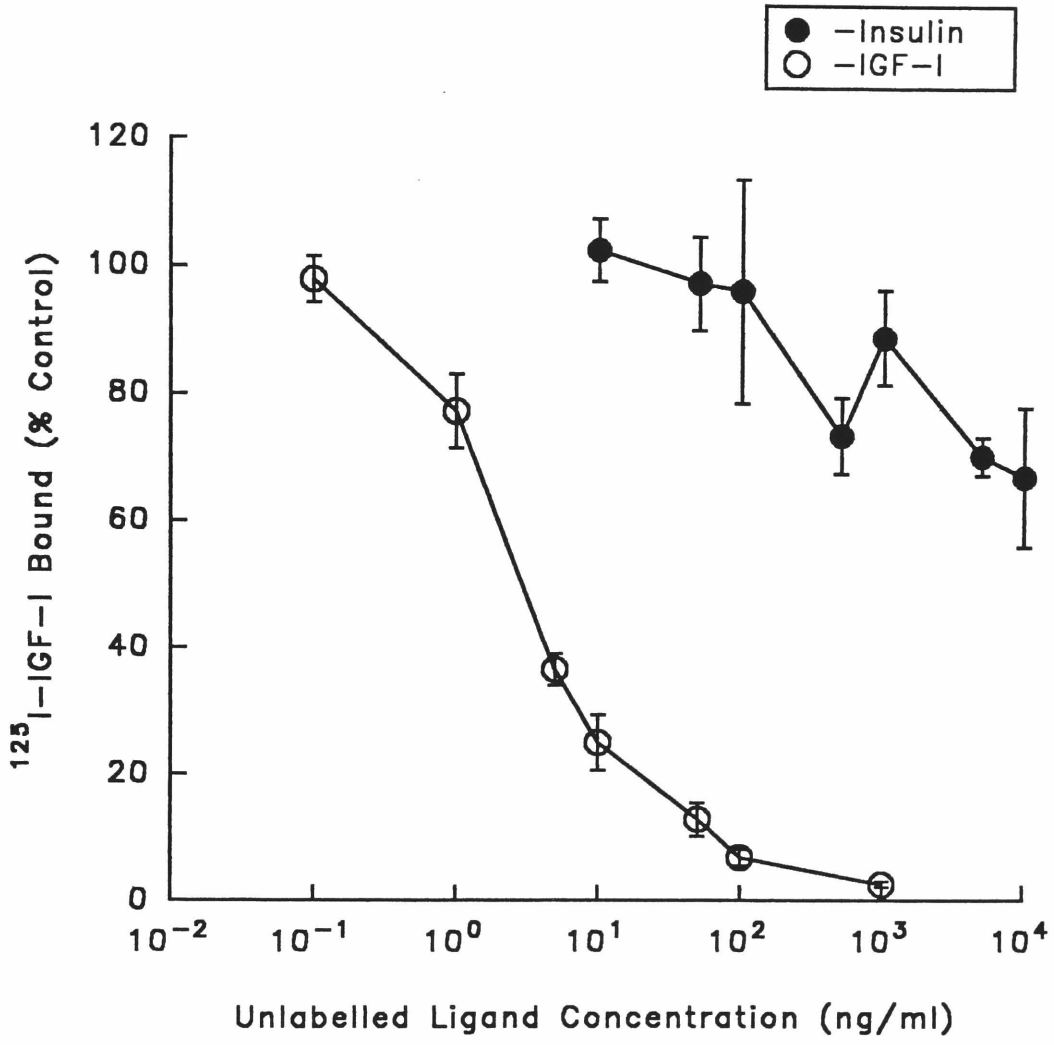


Figure 16. Competition by IGF-I and insulin for binding of ^{125}I-IGF-I to cultured human keratinocytes. ^{125}I-IGF-I (1 ng/ml) binding was measured in the presence of human recombinant insulin (0.01-10 µg/ml; Eli Lilly and Co.) or IGF-I (0.0001-1 µg/ml; Ethicon, Inc.). Keratinocytes grown in KGM were transferred to KBM for 1 d prior to the assay. Data are expressed as the percentage of counts bound in the presence of competitor/counts bound in KBM alone.

To assess potential IGF-I receptor mediated transmodulation of EGF receptor binding, ^{125}I-EGF binding to the EGF receptor was measured in cells that were first grown to near-confluence in KGM, then transferred for 24 h to KBM to remove the effects of exogenous growth factors, and finally incubated for 2 d with varying concentrations of insulin or IGF-I.

Figure 17A 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 (68), little or no decrease in EGF binding occurs. However at supraphysiologic concentrations where insulin activates the IGF-I receptor (68), insulin increases EGF binding to a maximum of more than 2-fold at an insulin concentration of 5 μg/ml (Figure 17A). 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 (84,85). 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, the effects of IGF-I on EGF receptor binding were assessed. The dose-response curve for EGF receptor binding in IGF-I treated keratinocytes is shown in Figure 17B. 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 (68). In the example given, IGF-I increased EGF binding by a maximum of 1.5-fold at a concentration of 100 ng/ml. Although the magnitude of the maximal increase shown in Figure 17 with IGF-I is less than that with

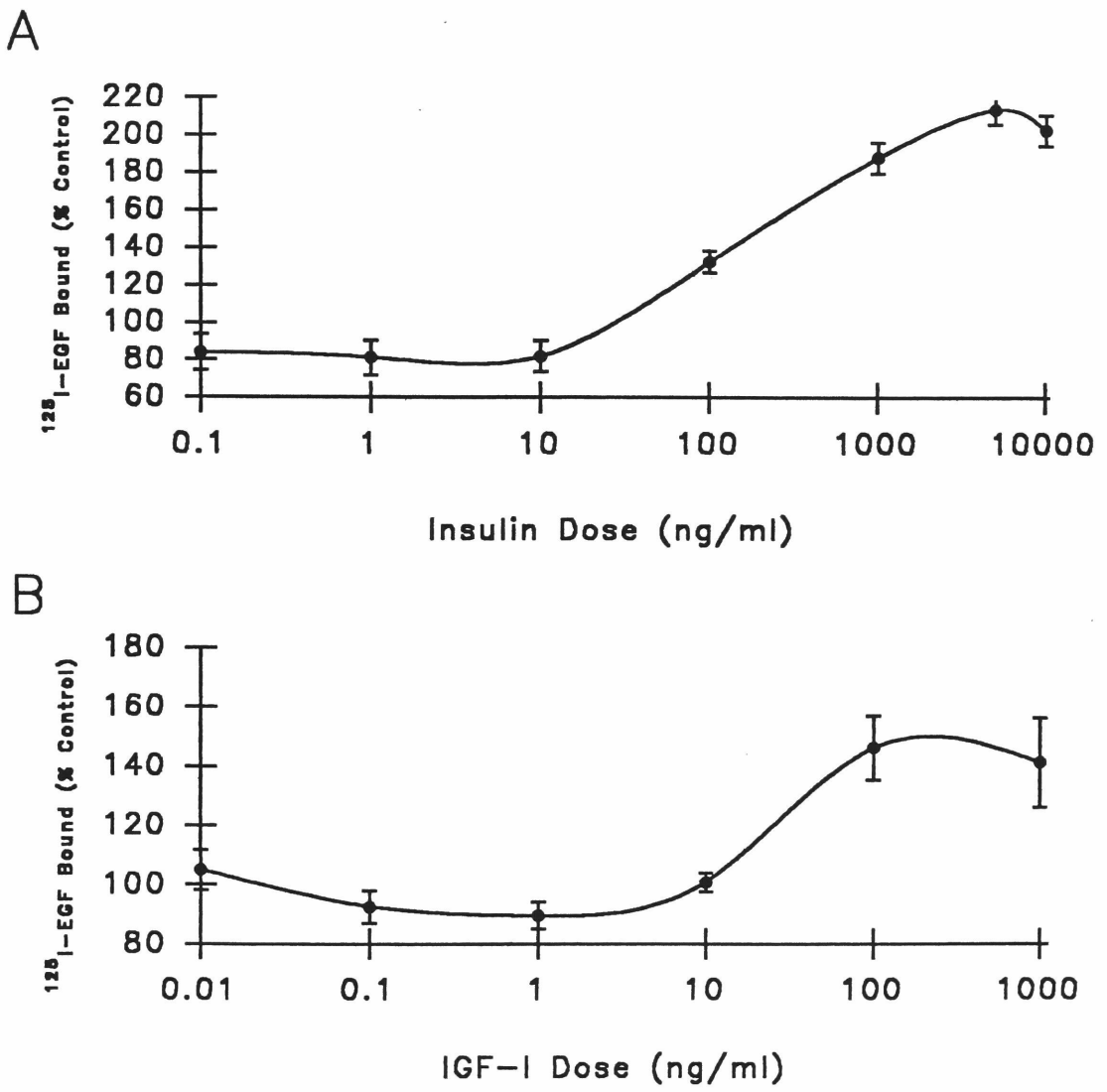


Figure 17. Dose-response of ^{125}I-EGF binding to human keratinocytes treated with human recombinant insulin or IGF-I. Keratinocytes grown in KGM were transferred to KBM for 1 d and then treated for 2 d with the indicated concentrations of human recombinant insulin (A) or IGF-I (B) in KBM. Values were 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.

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 among cell lines, but ranged from approximately 1.5- to 4-fold in 5 primary keratinocyte cell lines tested with an overall mean increase of 1.8-fold. 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 (86).

Figure 18 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 K_d of 1.8 nM. These cells possessed approximately 4.8 X 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-fold to 17 X 10^4 sites/cell. This result indicates that the increase in EGF receptor number observed in keratinocytes treated with KGM versus KBM (Figure 10) could be wholly attributable to effects of IGF-I or high-dose insulin on EGF receptor binding.

The increase in EGF binding caused by insulin was time dependent as shown in Figure 19. Treatment of cells over a 2 d 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 less than 1 min at 4°C followed by 6 h at 4°C in medium free of insulin was sufficient to cause an increase in EGF binding in comparison with cells in KBM alone.

The rapidity of the transmodulatory effect of IGF-I treatment on EGF receptor

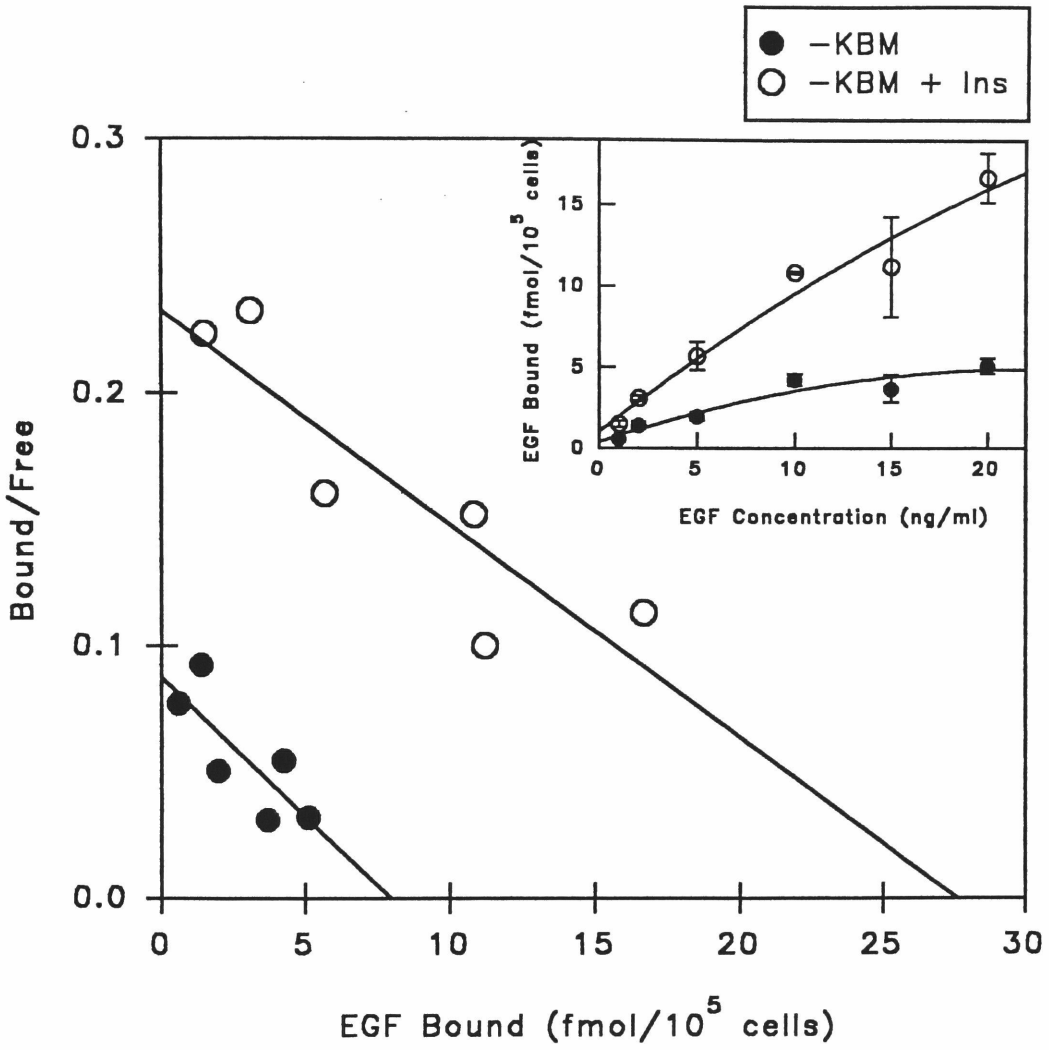


Figure 18. Scatchard analysis of ^{125}I-EGF binding to human keratinocytes treated with human recombinant insulin. Cells were treated as described in Figure 17 with 5 μg/ml human recombinant insulin in KBM (open circles) or KBM alone (closed circles) as control. The insert is a plot of EGF concentration added versus specific binding of the ligand. Values given represent mean specific binding of triplicate measurements in a single experiment.

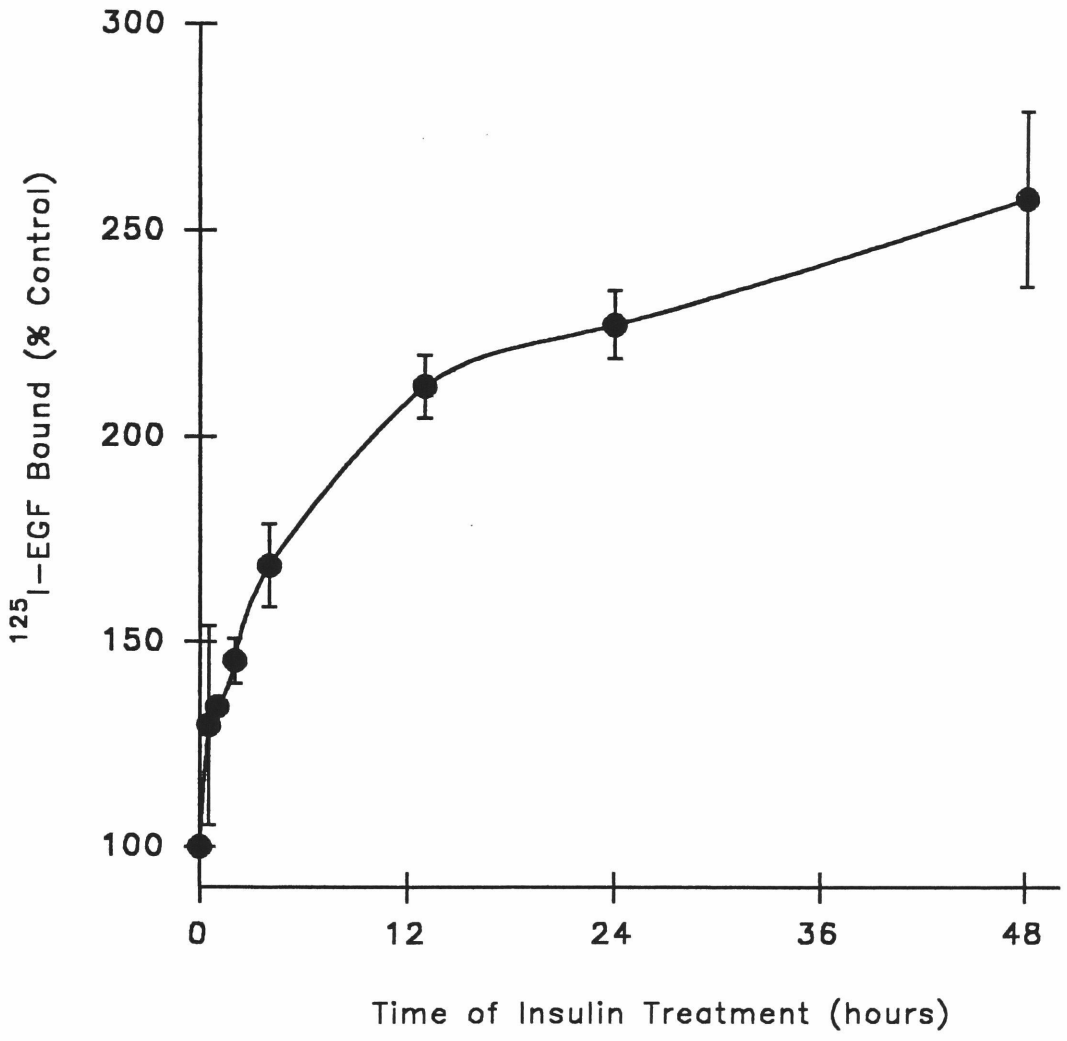
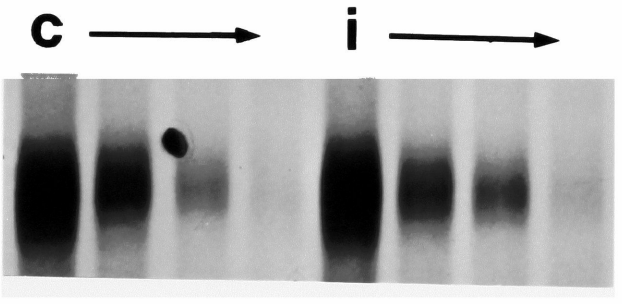


Figure 19. Kinetics of the increase in ^{125}I-EGF binding to human keratinocytes treated with insulin. Keratinocytes grown in KGM were transferred to KBM for 1 d prior to treatment with 5 μg/ml human recombinant insulin in KBM for up to 48 h. Early time points correspond to 0 min (no insulin addition), 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 ± standard deviation.

numbers would argue against this process being mediated by *de novo* synthesis of EGF receptors. In Figure 20, the possibility that this process might result from an alteration in EGF receptor half-life was tested. The figure demonstrates that cells treated with KBM alone versus KBM containing high-dose insulin showed no significant difference in EGF receptor half-life.

Figure 20. Insulin treatment of human keratinocytes does not affect EGF receptor half-life. Keratinocyte proteins were labeled with ^{35}S-methionine in KBM for 12 h prior to removal of the labeling medium at t=0. Cells were lysed immediately or further incubated for 12, 24, or 48 h in KBM alone (c) or containing 5 μg/ml bovine insulin (i). Samples were immunoprecipitated with rabbit anti-EGF receptor antibody and proteins were separated on a 7% polyacrylamide gel as described in Chapter 7. Following the direction of the arrows, keratinocytes were incubated for 0, 12, 24, or 48 h after the labeling period. Densitometric scanning of the gel revealed no change in EGF receptor half-life in control versus insulin-treated cells.


c
i

2.3 Discussion

In vivo, IGF-I mediates the growth promoting effects of growth hormone and is itself an important hormone in determining structure and function of adult tissues (59). While the primary function of IGF-I is to regulate growth-related cellular metabolism, it could also have effects on intracellular hexose transport and anabolic metabolism through cross reaction with the insulin or IGF-II receptors. IGF-I may be an especially important hormone for development and homeostasis of adult tissues, since its plasma level increases throughout adolescence to a peak concentration of about 40 nM and levels plateau at about 20 nM in adults (59). These concentrations are far above the K_d of the IGF-I receptor for IGF-I (about 1 nM), but IGF-I access to tissues is restricted by serum binding proteins for IGF-I. Tissue responses to IGF-I in adults may also be tightly regulated by IGF-I receptor expression, which decreases in many tissues throughout development and is at low levels in many adult tissues (64). Although IGF-I could potentially bind to insulin or IGF-II receptors in adult tissues, the mitogenic regulation of cellular growth is specifically mediated by the β subunit tyrosine kinase of the IGF-I receptor and not that of the insulin receptor (63). In this context, it is interesting that little change in IGF-I receptor expression is seen in basal epidermal keratinocytes of neonatal versus adult skin (Figure 4). The IGF-I receptor may thus play an important role in sustained proliferation of keratinocytes throughout life.

The localization of IGF-I receptors to the proliferative basal layer in normal epidermis supports the notion that interactions between the EGF and IGF-I receptor pathways could serve to modulate normal epidermal growth *in vivo*. Given the probable role of IGF-I as an epidermal mitogen, the restriction of IGF-I receptor expression to the basal epidermal compartment may largely limit proliferation to basal keratinocytes in normal skin. In this regard, IGF-I receptor localization is more specific for keratinocytes

with proliferative potential than is EGF receptor distribution, since EGF receptors can be detected at cell surface membranes of all viable epidermal layers ((48,72) and Figure 4). This suggests a potentially important difference in differentiation or developmental regulation of IGF-I and EGF receptor expression in human skin.

There may also be important differences in biochemical regulation of ligand-receptor binding interactions in the EGF and IGF-I receptors. *In situ* binding of ^{125}I-EGF is primarily limited to basal epidermal keratinocytes in epidermis, even though suprabasal keratinocytes also express EGF receptors. This finding suggests that regulation of the binding availability of existing EGF receptors may be an important determinant of receptor-ligand interactions *in vivo* (72). From the observation of diminished EGF binding to its receptor after treating keratinocytes with PMA, calcium, or ionophores, it appears that EGF receptor-ligand binding can be highly modulated in a negative fashion, possibly through PKC mediated threonine phosphorylation (77). Alternatively, increased cell surface EGF receptor number in cultured keratinocytes can be rapidly induced by an IGF-I receptor mediated transmodulation. In contrast, the ability of the IGF-I receptor to bind IGF-I is not negatively affected by PMA, calcium, or ionophore treatment of keratinocytes. Thus, expression of the IGF-I receptor is regulated differently from that of the EGF receptor in cultured keratinocytes. These data, combined with details of the IGF-I receptor gene promoter sequence (87) and the pattern of IGF-I receptor expression in epidermis, suggest that IGF-I receptor-ligand interactions in tissue may be more a function of differentiation-specific control of receptor expression rather than biochemical modification of ligand binding potential. The finding that binding of ^{125}I-IGF-I to epidermal tissue *in situ* closely parallels IGF-I receptor expression as defined by immunohistochemistry fulfills one prediction of this IGF-I receptor-ligand binding scheme.

The results described in this chapter 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 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 (31,32,35,38), but none have clearly demonstrated minimal growth factor requirements to sustain spreading and proliferation of human keratinocytes in chemically defined medium. The data presented here on human keratinocyte growth agree with those on mouse keratinocytes which demonstrate synergistic enhancement of DNA synthesis induced by IGF-I in combination with EGF or bFGF (65) or by insulin in combination with EGF (88).

At low cell inoculation densities used in these 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 are accomplished in a complete growth medium (20,24,25,68,89). 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 (20,24,25,68,89). When the effects of EGF and IGF-I or insulin on keratinocyte growth were assayed 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 fold in serum-free medium (89) and fibronectin or other connective tissue molecules can increase cell proliferation under these conditions (89,90).

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 (88). 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 these experiments. Studies of EGF receptor binding in normal human keratinocytes show that the antiproliferative agents IFN-γ, TGF-β, and CSA decrease EGF receptor binding to 25-50% of untreated control levels (91). Thus, the 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. The mechanism by which IGF-I transmodulates the EGF receptor is not known. However, the rapid time course of the process and the lack of alteration in EGF receptor half-life would seem to argue for an alteration in trafficking of preexisting EGF receptors. In this context, it is noteworthy that existence of a "latent" pool of EGF receptors in human skin has been proposed previously based on the observation that immunohistochemically defined EGF receptors are found throughout the viable layers of the epidermis, while radioligand binding studies show a more basalar distribution of EGF ligand binding sites (48,72).

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 (29)). .On the basis of these observations, it has been postulated that growth factors act at specific, sequential points in the cell cycle to allow continued growth (55). 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. Since keratinocytes lack PDGF receptors and are thus not directly responsive to PDGF (see Chapter 4), growth of connective tissue and epithelial cell types may be regulated by fundamentally different sets of growth factors and intracellular signaling pathways. Insulin-like factors appear to be obligate for keratinocyte colony growth, but only if EGF,

FGF, or a pituitary extract is simultaneously present (33,35). It has been proposed that IGF-I may act as a progression-like factor since it can stimulate proliferation of keratinocytes previously exposed to serum (68). 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.

The increased expression of IGF-I receptors in suprabasal keratinocytes of lesional psoriatic skin could be important in regulating the keratinocyte hyperplasia associated with this disorder. IGF-I receptor distribution closely parallels the increased size of the keratinocyte proliferative compartment in active psoriatic epidermis (6). The potential need for IGF-I receptor activation to trigger keratinocyte proliferation might help to explain the relative confinement of keratinocyte proliferation to basal and suprabasal epidermal layers in psoriasis, despite increased expression of TGF-α (26,27) and EGF receptors (48,72) capable of ligand binding over all viable epidermal layers in psoriasis. Indeed, mitogenic responsiveness to IGF-I in some epithelial cell types appears to be controlled through regulation of cell surface IGF-I receptors and not the available ligand concentration (92). Even so, free IGF-I would need to be available to activate IGF-I receptors in psoriatic skin if this receptor system is relevant to psoriatic epidermal hyperplasia. A relative abundance of IGF-I might be available to psoriatic epidermis through leaky, fenestrated capillaries in the papillary dermis (93), or by IGF-I secreted in a paracrine fashion from dermal fibroblasts which overexpress PDGF receptors in active psoriatic lesions (see Chapter 4). There is even the possibility that IGF-I could be supplied to the epidermis in an autocrine fashion, since IGF-I immunoreactivity can be induced in the epidermis under some conditions (66). The ability to detect IGF-I receptor kinase activity in psoriatic lesional keratinocytes in the absence of exogenously added IGF-I indicates that the IGF-I receptor pathway is activated in psoriatic epidermis. A role for participation of the IGF-I receptor-ligand system in the pathogenesis of psoriasis is suggested from clinical improvement produced by somatostatin-like drugs that diminish

plasma IGF-I concentrations (94). Furthermore, activation of the increased IGF-I receptors present in lesional psoriatic skin might increase EGF receptor expression (a characteristic of psoriatic epidermis) via IGF-I mediated transmodulation of the EGF receptor.

Chapter 3: The FGF Receptor in Cultured Human Keratinocytes and in Normal and Psoriatic Skin

3.1 Introduction

FGF-related peptides constitute a family of heparin-binding growth factors with angiogenic properties and mitogenic activity for a variety of cell types including keratinocytes (95). There are at least seven members of this hormone family including aFGF, bFGF, Kaposi FGF, FGF5, FGF6, and KGF (96-100). All of these proteins have mitogenic activity for fibroblasts with the exception of KGF, which appears to be a specific mitogen for epithelial cells including keratinocytes (100). KGF is synthesized by dermal fibroblasts and may act as a paracrine modulator of epidermal growth (101). Keratinocyte lines exhibit varying degrees of growth activation by particular FGF-related peptides depending upon the cell line and culture conditions (33,34,84,102-104). Additionally, bFGF has been identified as an active component of growth stimulating pituitary extracts used in standard serum-free keratinocyte culture systems (96). Cultured human keratinocytes synthesize bFGF mRNA (105); however, bFGF and aFGF proteins lack signal peptides so bFGF may not be released from keratinocytes except in response to tissue damage (106). Some researchers have suggested that bFGF may still be able to exert autocrine growth stimulation by binding and activating receptors within the cell ("intracrine" activation) or that bFGF may be released into the extracellular matrix by unknown mechanisms where it may act as a "reservoir" of ligand available for release to FGF receptors (107).

FGF receptors constitute an equally diverse family of tyrosine kinase cell-surface receptors possessing two or three extracellular immunoglobulin-like domains which may function in ligand binding (108). Not only have multiple FGF receptor genes been

identified, but alternatively spliced mRNAs from individual genes may be translated into as many as twelve different polypeptides (109). Chemical crosslinking experiments have identified FGF receptor species with molecular weights ranging from approximately 100 to 165 kd (110,111). The function of these multiple receptor forms and genes is uncertain and is further complicated by the finding that FGF receptors appear to bind more than one FGF-related peptide with similar high affinity and, thus, may not be specific to individual ligands (110). A human keratinocyte FGF receptor has been cloned from a cDNA library (112), but FGF receptor proteins have not been identified in normal human keratinocytes. This chapter provides preliminary data regarding the identification of an FGF receptor protein in cultured normal human keratinocytes and FGF receptor distribution in normal and psoriatic skin. Taken together with studies of the growth stimulating properties of FGF-related peptides, these findings suggest that the FGF receptor pathway may contribute to keratinocyte growth regulation.

3.2 Results

3.2.1 IGF-I and bFGF Act Synergistically to Promote Growth of Cultured Human Keratinocytes

To analyze the function of bFGF in normal keratinocyte growth, the growth assay described in Figures 2 and 3 of Chapter 2 was extended to the study of keratinocytes grown in the presence of various combinations and concentrations of bFGF and IGF-I (Table 1). Keratinocytes grown in the presence of bFGF alone showed only a minimal increase in cell number after 6 d, as was the case with IGF-I alone (Figures 2 and 3, and Table 1). In contrast, as was found with the combination of EGF and IGF-I (Figures 2 and 3), bFGF and IGF-I together showed a marked synergistic enhancement of cell growth. Growth of keratinocytes in the presence of both bFGF and EGF had an additive effect on cell number with no apparent growth synergy (data not shown). The results described in Table 1 reinforce the findings in Chapter 2 indicating that activation of the IGF-I receptor pathway is critical for keratinocyte proliferation. Preliminary findings indicate that growth stimulation by aFGF and KGF is also dependent on IGF-I receptor activation in cultured human keratinocytes (not shown).

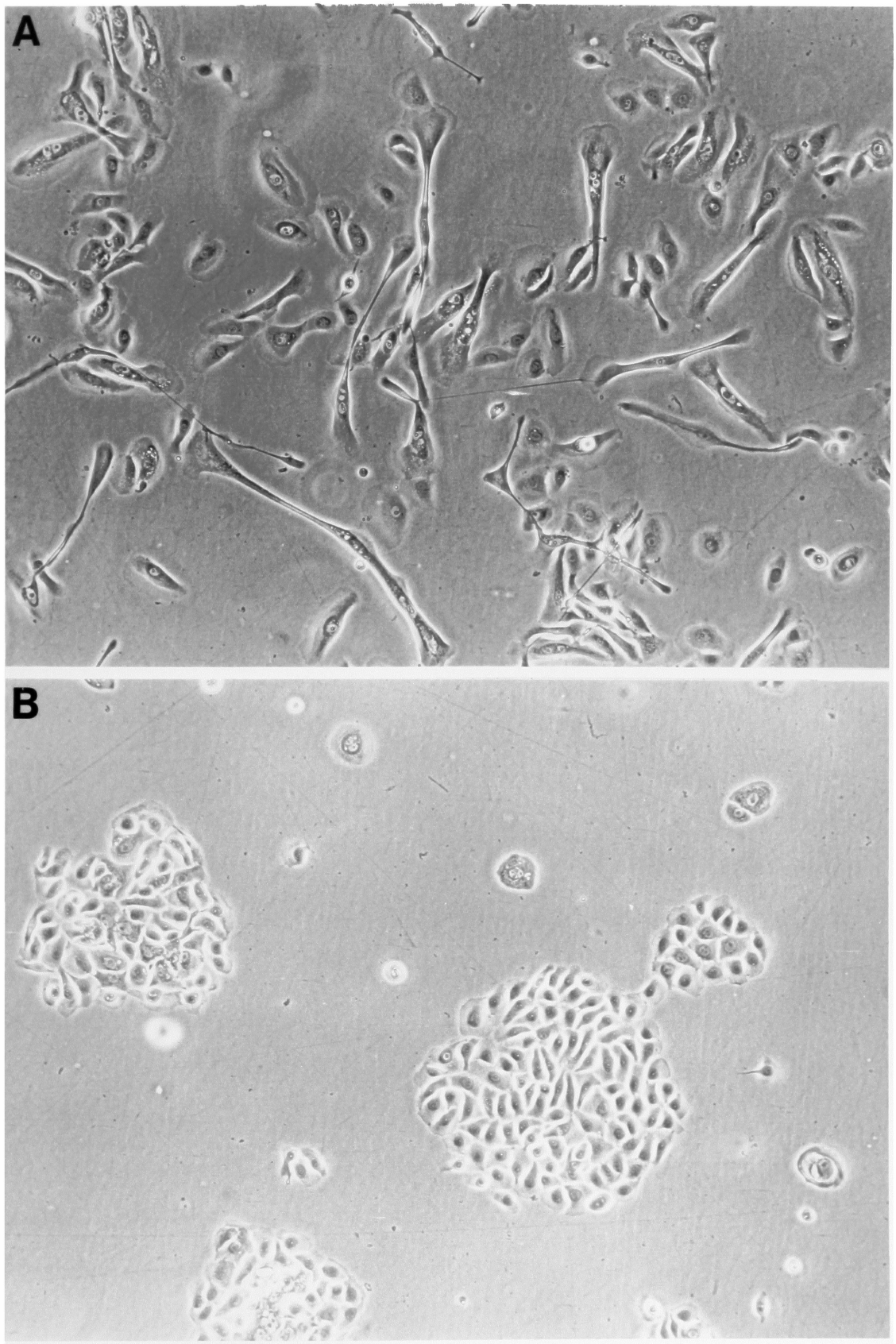
Table 1. -- Basic FGF Acts Synergistically with IGF-I to Promote Keratinocyte Proliferation*

Growth Factor Added	**Cell Number**
None	15,500 ±1360
IGF-I	27,700 ± 4600
bFGF	23,900 ± 2460
IGF-I + bFGF	121,000 ± 4540

* There were 35,000 keratinocytes present in each 25-cm^2 culture plate at the initiation of the experiment. The basal medium was KBM/HC. IGF-I (Pepro Tech Corp.) was used at 100 ng/ml. Basic FGF (Synergen) was added at 50 ng/ml. After 6 d, cell counts were measured using an electronic counter.

Despite the finding that EGF and bFGF act similarly in combination with IGF-I to promote keratinocyte growth, in other respects the effects of EGF and bFGF upon keratinocytes are distinct. Figure 21 shows the typical morphology of keratinocytes grown in the presence of EGF and IGF-I (Figure 21A) or bFGF and IGF-I (Figure 21B). Colonies of keratinocytes grown in the presence of EGF consist of loosely associated cells with an elongated appearance, while keratinocytes cultured in the presence of bFGF formed densely-packed, compact colonies.

Figure 21. Keratinocyte morphology in the presence of different growth factors. Keratinocytes seeded and grown in KBM/HC containing IGF-I/EGF (A) or IGF-I/bFGF (B) were photographed after 6 d. Recombinant human EGF, bFGF, and IGF-I were all added at 100 ng/ml.


A
B

3.2.2 Keratinocytes Express a Single Major bFGF Receptor Protein Based upon Chemical Crosslinking

While FGF-related peptides have been shown to stimulate human keratinocyte growth (33,34,102), no FGF receptor protein has yet been identified in normal human keratinocytes. To identify such a protein, radioiodinated bFGF was chemically crosslinked to intact cultured keratinocytes as shown in Figure 22. Addition of the crosslinking agent disuccinimidyl suberate to keratinocytes preincubated with ^{125}I-bFGF resulted in crosslinking of the iodinated ligand to a single major species with an approximate molecular weight of 160 kd (assuming that each receptor molecule binds a single bFGF molecule) (Figure 22, lane 1, arrow). This protein was not seen in the absence of crosslinker (Figure 22, lane 2) or in the presence of excess quantities of unlabelled bFGF (Figure 22, lanes 3-5). In lanes 3-5, the lower molecular weight species presumably represent multimeric forms of bFGF. The molecular weight of the receptor identified by this experiment is consistent with the size of the largest FGF receptor molecules identified in other cell types (110).

To confirm the identification of a keratinocyte FGF receptor, lysates of cultured keratinocytes and shave biopsy specimens from uninvolved and involved psoriatic skin were immunoblotted with a rabbit polyclonal antibody directed against the human *flg*-related FGF receptor (Figure 23). The FGF receptor antibody recognized a $M_r \approx 165$ kd protein in both cultured keratinocytes (Figure 23, lane 1) and in epidermal tissue specimens (Figure 23, lanes 2 and 3). This finding is consistent with this protein being the receptor identified by ligand crosslinking, although it could also be a related FGF-family receptor. Immunoblotting with the FGF receptor antibody detected no consistent alteration in FGF receptor protein levels in 3 paired lesional and non-lesional psoriatic shave biopsies (not shown).

Figure 22. Chemical crosslinking of ^{125}I-bFGF to intact cultured human keratinocytes. Keratinocytes were incubated for 2 h at 4°C with 10 ng/ml ^{125}I-bFGF alone (lanes 1 and 2) or in the presence of three different sources of excess recombinant bFGF: 500-fold excess Pepro Tech bFGF (lane 3), 5,000-fold excess Synergen bFGF (lane 4), or 5,000-fold excess Calbiochem bFGF (lane 5). Crosslinking with 1 mM disuccinimidyl suberate was performed for 20 min at 4°C. The sample shown in lane 2 did not receive any crosslinker. Keratinocytes were lysed and samples were separated on a 7.5% polyacrylamide gel and the dried gel was autoradiographed. A single major species of M_r ≈160,000 kd (after subtracting the molecular weight of the ligand) can be seen in lane 1. No bands are seen in the absence of crosslinker (lane 2) and only low molecular weight bands, probably corresponding to crosslinked ligand, are visible in lanes 3-5.

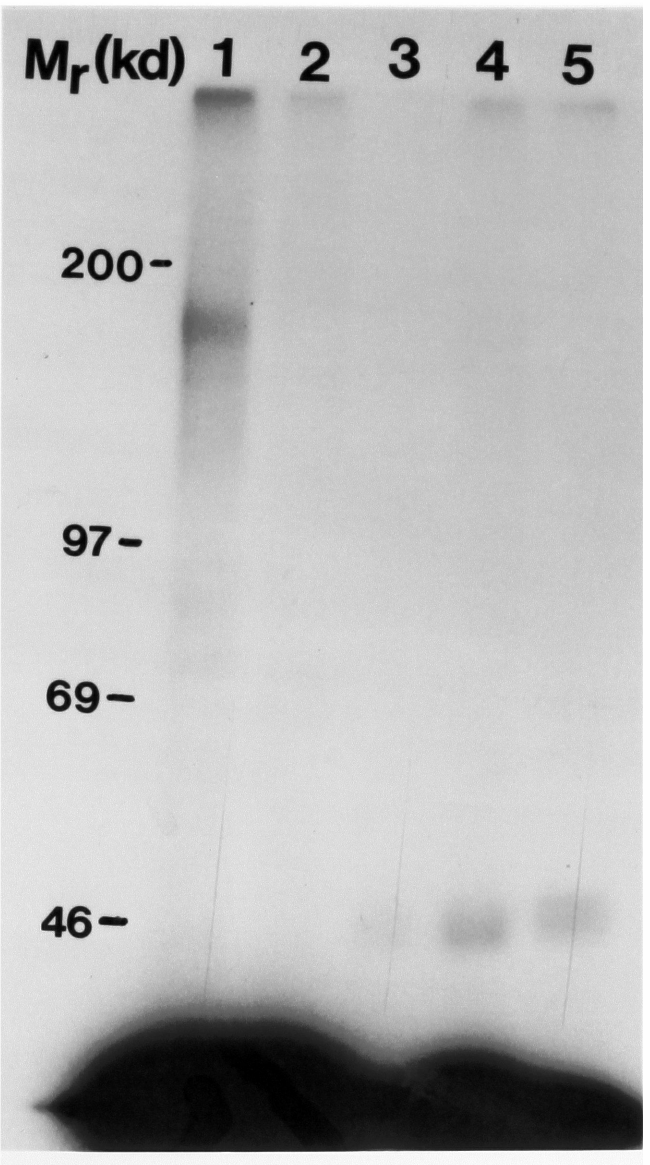
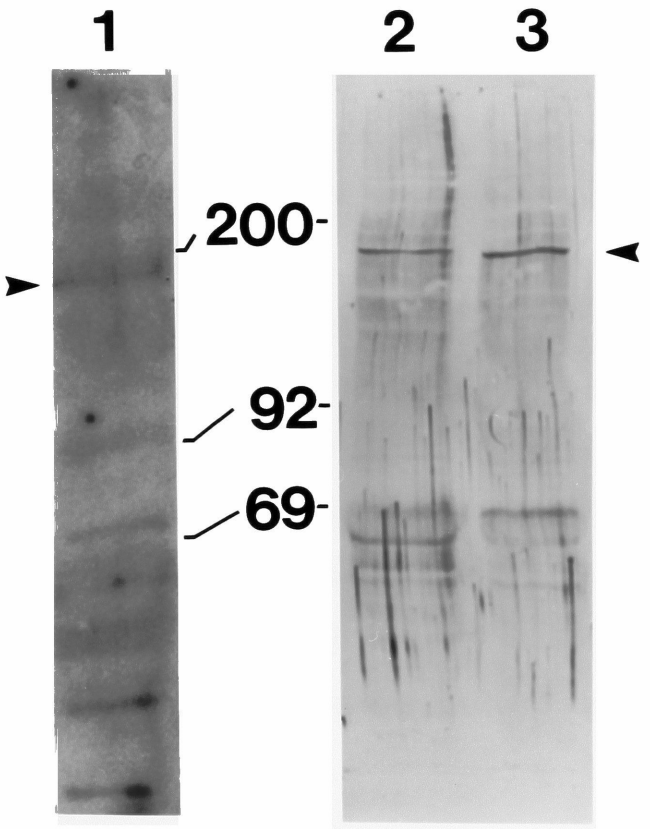

M_r(kd) 1 2 3 4 5
200-
97-
69-
46-

Figure 23. Immunoblotting of a polyclonal rabbit anti-FGF receptor antibody. Proteins were separated by SDS-PAGE and then transferred to nitrocellulose. Samples were obtained as described in Chapter 7 from cultured keratinocytes (lane 1) and from epidermal and papillary dermal tissue. Skin samples were from a non-lesional (lane 2) and a lesional (lane 3) biopsy of a patient with psoriasis. A major protein band of approximately 165 kd was detected in all three specimens as well as a series of lower molecular weight minor bands.


1
2
3
200-
92-
69-

3.2.3 Competition for Radioactive Ligand Binding by FGF-related Peptides

To test the relative binding affinities of various FGF-related peptides for the identified keratinocyte FGF receptor, competition experiments were performed in which excess quantities of unlabelled aFGF, bFGF, and KGF were allowed to compete for binding of ^{125}I-bFGF to cultured keratinocytes. As seen in Figure 24 (lower), 2 different recombinant sources of bFGF competed most effectively with the radioligand. The order of competition for binding was bFGF > KGF > aFGF. Since FGF molecules can bind matrix with low affinity, the experiment was also conducted with a 2 M NaCl wash to remove binding to these low affinity sites (107,113). Under these conditions, ~ 90% of control binding was abolished but the relative order of radioligand competition by the unlabelled ligands was preserved (data not shown).

In a parallel experiment, competition for binding of ^{125}I-KGF was assessed (Figure 24, upper). In this experiment, bFGF and KGF were able to compete equally well for radioligand binding while aFGF competed less successfully. This experiment is consistent with KGF binding to the same receptor as bFGF rather than its own distinct receptor on cultured human keratinocytes. However, the identity of the receptor to which KGF binds in cultured normal human keratinocytes remains uncertain.

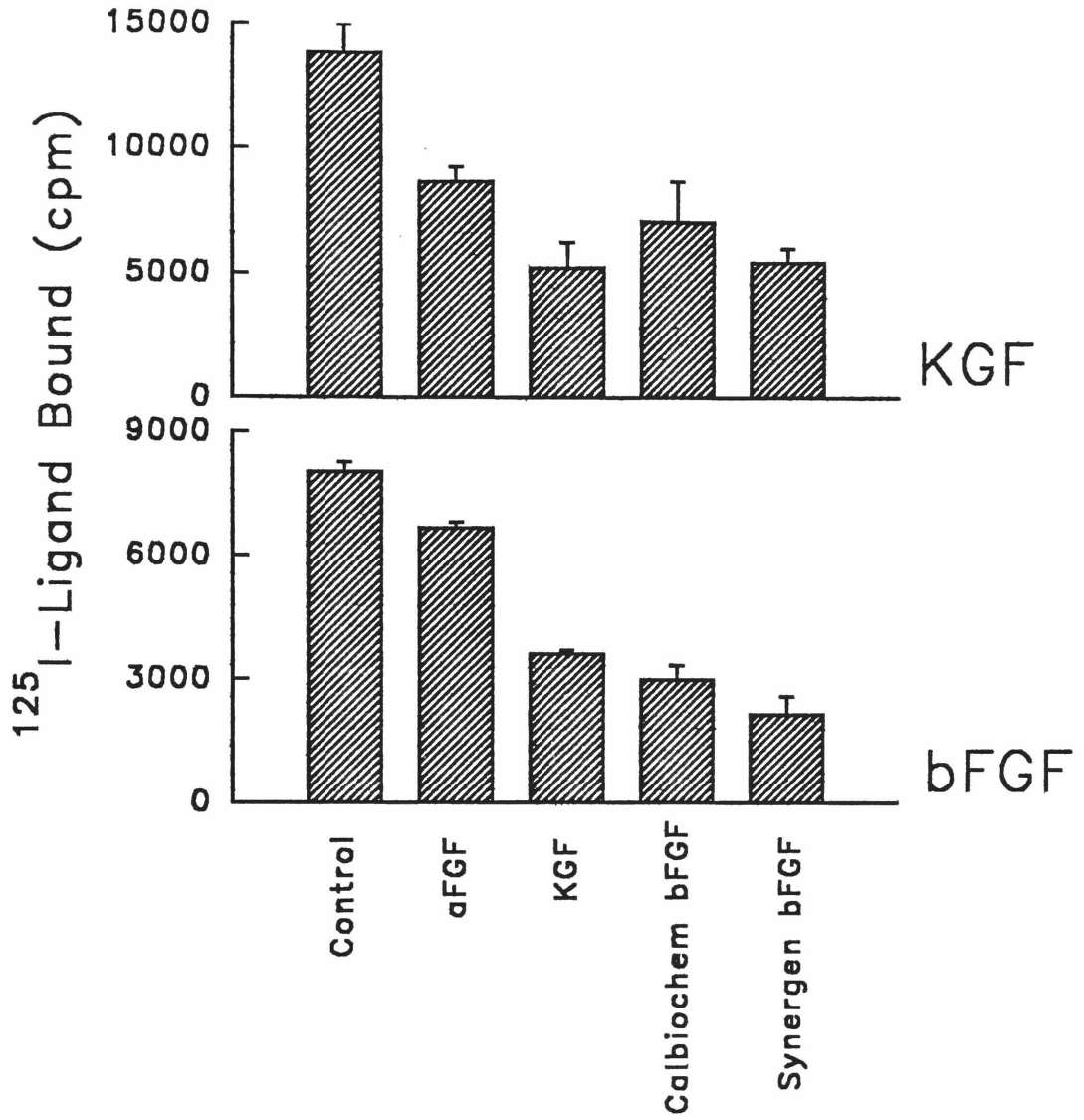
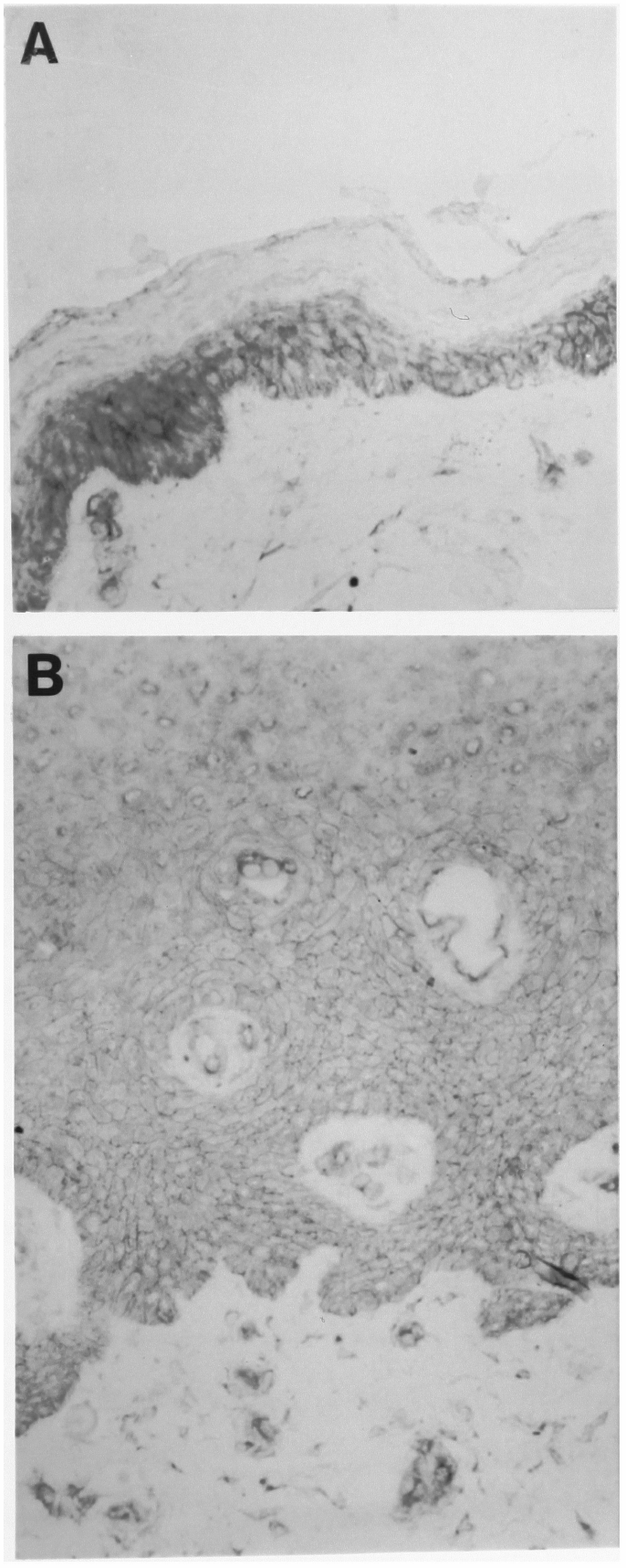


Figure 24. Competition for ^{125}I-bFGF and ^{125}I-KGF binding to cultured human keratinocytes. Keratinocyte binding of radioiodinated KGF (top) or bFGF (bottom) was measured in the absence of competitor (control) or in the presence of a 5,000-fold excess of the indicated recombinant human peptides. In a separate experiment (not shown), addition of EGF and IGF-I along with radioisotope did not compete for binding by the radioactive ligand.

3.2.4 Immunohistochemical Staining for the FGF Receptor in Human Skin

As was the case with EGF and IGF-I receptor localization, it would be expected that synergistic growth factor interactions between the IGF-I and FGF receptors would demand that both receptor systems be expressed within the proliferative cell compartment of the epidermis. The FGF receptor antibody that identified a keratinocyte FGF receptor species by Western blotting in Figure 23 was used to analyze FGF receptor distribution in uninvolved and lesional psoriatic skin (Figure 25). Plasma membrane staining for the FGF receptor was detected on keratinocytes located throughout the viable layers of the epidermis (Figure 25A). This finding indicates that IGF-I and bFGF can potentially act in synergy within the basal pool of proliferative keratinocytes. Moreover, it reconfirms the finding observed with EGF and IGF-I receptor localization that the IGF-I receptor is the growth factor receptor most closely associated with defining the proliferative keratinocyte pool. In Figure 25B, psoriatic lesional epidermis is also seen to have plasma membrane staining for the FGF receptor throughout the viable cell layers. Further study will be needed to determine if the less intense staining of lesional epidermis in this sample is representative of altered FGF receptor expression in psoriasis.

Figure 25. Immunohistochemical staining of human skin with a rabbit anti-FGF receptor antibody. Sections from normal appearing, uninvolved psoriatic skin (A) and lesional psoriatic skin (B) were stained by the immunoperoxidase technique using the same antibody that recognized the 165 kd protein by Western blotting in Figure 23. Membrane staining of all viable epidermal layers can be seen in both the normal and psoriatic specimens.


A
B

3.3 Discussion

The results in this chapter establish that mitogenic stimulation of cultured human keratinocytes by bFGF is dependent upon the simultaneous presence of IGF-I receptor activation. The synergistic stimulation of growth exhibited by bFGF with IGF-I is similar to that described for EGF and IGF-I in Chapter 2. Experimentation thus far indicates that IGF-I receptor stimulation is absolutely required for the stimulation of keratinocyte proliferation by FGF-related peptides; however, it is possible that other FGF-related peptides could act through distinct mechanisms which would not require IGF-I receptor activation. Along with the observation that EGF and bFGF do not synergize with each other (84,102,104), these findings support a model for cell cycle progression in keratinocytes where FGF and EGF act at the same point in the cell cycle, distinct from the action of IGF-I. While EGF and FGF may act similarly with regard to the cell cycle, they have distinct effects on keratinocyte morphology in culture and time-lapse photography indicates that EGF and IGF-I treated keratinocytes exhibit far greater motility than bFGF and IGF-I treated keratinocytes (H. Uyttendaele, personal communication).

The effects of bFGF on keratinocyte proliferation in cultured human keratinocytes appear to be mediated exclusively by specific interaction with a 160 kd FGF receptor protein. The size of this protein indicates that it may belong to the class of FGF receptors having three extracellular immunoglobulin-like domains. Radioreceptor competition experiments suggest that the FGF receptor identified by crosslinking preferentially binds bFGF over aFGF and KGF. The particular order of binding affinity has not been previously described and could be indicative of the expression of an FGF receptor species unique to human keratinocytes. The lack of crosslinking of bFGF to any other receptor could indicate that human keratinocytes express a single class of FGF receptors. However, bFGF has been noted to bind with 20-fold less efficiency than KGF to the KGF

receptor in Balb/MK cells (114). FGF receptors which bind other FGF-related peptides, but not bFGF, may still be identified and the expression of other FGF receptor species may be detectable by other culture conditions.

A protein of similar size to that identified by crosslinking was detected with an FGF receptor specific polyclonal antibody. While this protein may be a related member of the FGF receptor family, the size of the protein is consistent with its being identical to the receptor identified by crosslinking. Immunohistochemical staining of lesional and uninvolved psoriatic epidermis with this polyclonal antibody suggests that IGF-I and FGF receptor pathways could act simultaneously upon cells within the proliferative compartment of the epidermis to support keratinocyte growth. FGF molecules in the epidermis could be derived from keratinocyte bFGF synthesis, fibroblast KGF synthesis, or serum derived FGF (101,105). Additionally, bFGF from the epidermis could possibly act on dermal fibroblasts and capillary endothelial cells, especially in response to tissue injury during wound healing or as part of the Koebner reaction in psoriasis. Further study will be necessary before it can be established whether regulation of components of the FGF receptor pathway is disrupted in psoriatic skin.

Chapter 4: The PDGF Receptor in Cultured Human Keratinocytes and in Normal and Psoriatic Skin

4.1 Introduction

PDGF is a potent mitogen for dermal fibroblasts, smooth muscle cells, and other types of connective tissue cells. PDGF is also chemotactic for fibroblasts, monocytes, and neutrophils (reviewed in (115)). PDGF stimulates collagen and collagenase synthesis by fibroblasts *in vitro* as well as the production of IGF-I (116). These properties and the fact that PDGF is the most abundant growth factor in platelets (115) suggest that PDGF might be a major regulator of fibroplasia and connective tissue growth in conditions such as healing wounds or psoriasis. Since PDGF is also produced by some epithelial cell types (117), including human keratinocytes (23,118), PDGF might also affect function of dermal connective tissue cells by release from epidermal keratinocytes or other epithelial cells.

PDGF is composed of two chains, A-chain and B-chain, which dimerize to form AA, AB, and BB. All three forms are biologically active and have been identified from natural sources. Cellular responses to PDGF are dependent on the interaction of PDGF with cell surface receptors. Two PDGF receptor molecules have been identified, termed α and β, and cDNA clones have been obtained for each (reviewed in (119)). Both of the receptors contain extracellular ligand-binding domains and intracellular split tyrosine kinase domains. It has recently been proposed that dimerization of the two receptor subunits is required for the generation of high affinity binding sites, and that the three receptor dimers (α/α, α/β, β/β) have different PDGF ligand binding specificities (reviewed in (119)). The ability of a cell to respond to a particular form of PDGF appears to be determined by the amount and ratio of the two receptor subunits present on the cell surface. It is still unclear whether unique pathways exist for the generation of intracellular

signals for the two receptor subunits.

Cultured psoriatic fibroblasts have been previously shown to have an increased sensitivity to the mitogenic and chemotactic properties of PDGF suggesting that some component of this growth factor pathway may be altered in hyperproliferative skin (120). Conversely, PDGF receptors have not been described in human keratinocytes. Since growth studies in connective tissue cells indicate that PDGF is a major mitogenic "competence" factor (29,55), a lack of keratinocyte PDGF receptors would indicate that keratinocyte growth is modulated in a fundamentally different manner from fibroblast growth. The work described in this chapter was designed to establish whether or not keratinocytes express PDGF receptors and to examine the pattern of PDGF receptor expression in psoriasis and chronic wounds, two conditions associated with increased fibroplasia and vascularization as well as epidermal hyperplasia (30).

4.2 Results

4.2.1 Cultured Keratinocytes Do Not Express Functional PDGF Receptors on Their Cell Surfaces

To assess whether keratinocytes express functional PDGF receptors, normal human keratinocytes and fibroblasts were treated with PDGF for 0, 1 or 3 h. Such treatment causes cell surface receptor "down-regulation" in cells expressing biologically active PDGF receptors that are available for ligand binding (121). The course of this process was followed by measuring binding of radioiodinated monoclonal antibodies recognizing the α and β subunits of the PDGF receptor. As shown in Figure 26, binding of both antibodies decreased by >50% in fibroblasts after 3 h treatment with PDGF-BB reflecting PDGF receptor internalization. In contrast, keratinocytes bound far fewer counts initially and this background level of binding was unchanged by PDGF treatment. This finding indicates that keratinocytes cultured under these conditions do not express active PDGF receptors and would thus not be directly responsive to autocrine PDGF secretion.

4.2.2 Increased Dermal Expression of PDGF Receptors in Growth Activated Skin Wounds and Psoriasis

Epidermis adjacent to chronic skin wounds and epidermis from active psoriatic plaques display markers of regenerative maturation, a form of epidermal growth activation associated with epidermal hyperplasia and wound repair (8,122). Both conditions also display activation of dermal fibroblasts, increased fibroplasia, and increased

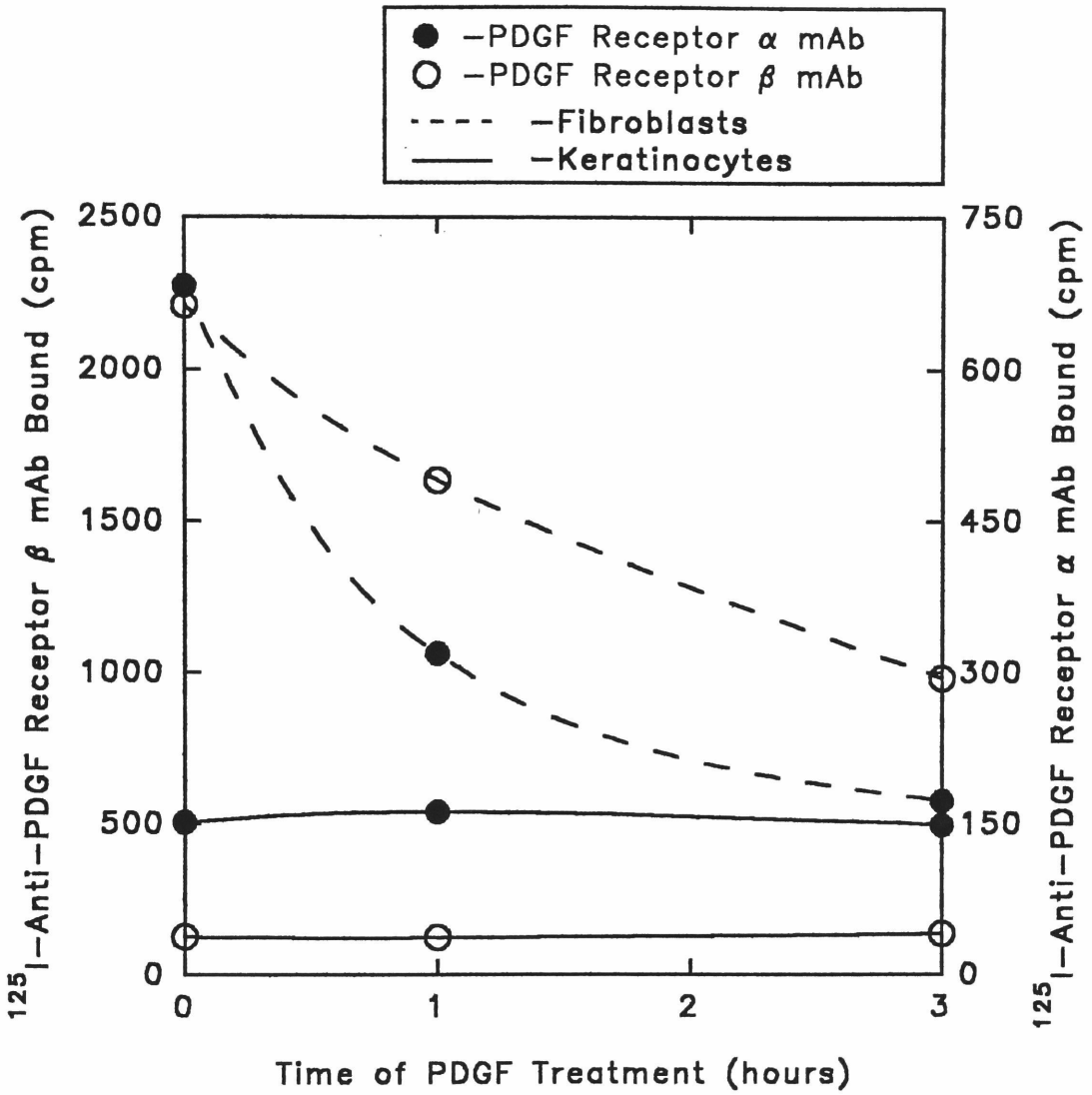


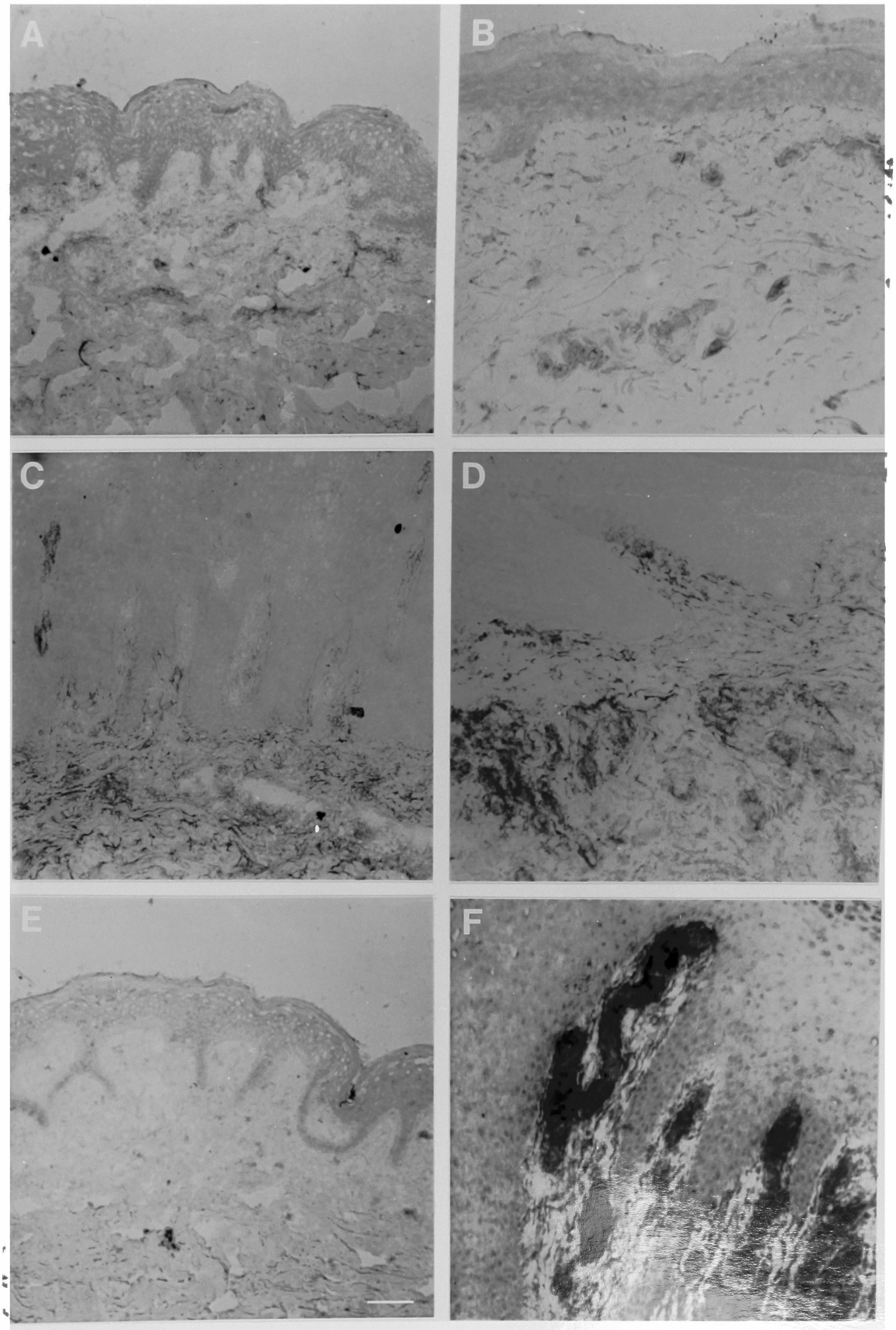
Figure 26. "Down-regulation" of PDGF receptors on cultured cells in response to PDGF treatment. Cultured normal human keratinocytes (solid lines) or fibroblasts (dashed lines) were treated with 50 ng/ml PDGF-BB for 0, 1, or 3 h. Receptor down-regulation in response to ligand binding was assayed by measuring the binding to cells of radioiodinated mAbs to the PDGF receptor α (closed circles) and β (open circles) subtypes. Keratinocytes bound low levels of both antibodies which did not change with PDGF treatment. In contrast, fibroblasts exhibited diminished binding with time of PDGF treatment indicative of progressive receptor down-regulation.

neovascularization or vascular growth.

Since previous work has shown that cultured psoriatic fibroblasts are more responsive to the chemotactic and mitogenic properties of PDGF (120) and that PDGF is beneficial in the treatment of acute wounds (123), potential alterations in PDGF receptor expression in growth activated skin were assessed. Expression of the PDGF receptor β-subunit was studied in full thickness skin biopsies from 10 patients with psoriasis and from 15 patients with chronic lower extremity wounds. In each case, expression of the PDGF receptor was examined on cryostat-prepared sections of normal and lesional skin using the monoclonal antibody PR7212, which has been extensively characterized (124). In normal skin, faint staining of dermal fibroblasts and vascular elements was noted with this antibody (Figure 27B). Non-lesional skin from psoriatic patients showed a similar expression pattern (Figure 27A). No specific epidermal staining for the PDGF receptor was seen in normal epidermis or non-lesional epidermis from psoriatic patients (Figures 27A and 27B). A control mouse IgG produced no staining of epidermal or dermal structures (Figure 27E). Analysis of skin from active psoriatic lesions (Figure 27C) and chronic wounds (Figure 27D and 27F) showed a marked increase in staining intensity of dermal fibroblasts and vascular elements (Figure 27F) in comparison to normal skin from these conditions. The hyperplastic epidermis associated with these lesional biopsies failed to show staining for the PDGF receptor, consistent with the *in vitro* findings described in Figure 26 as well as the work of other investigators indicating that keratinocytes do not express functional PDGF receptors or synthesize PDGF receptor mRNA (30,118).

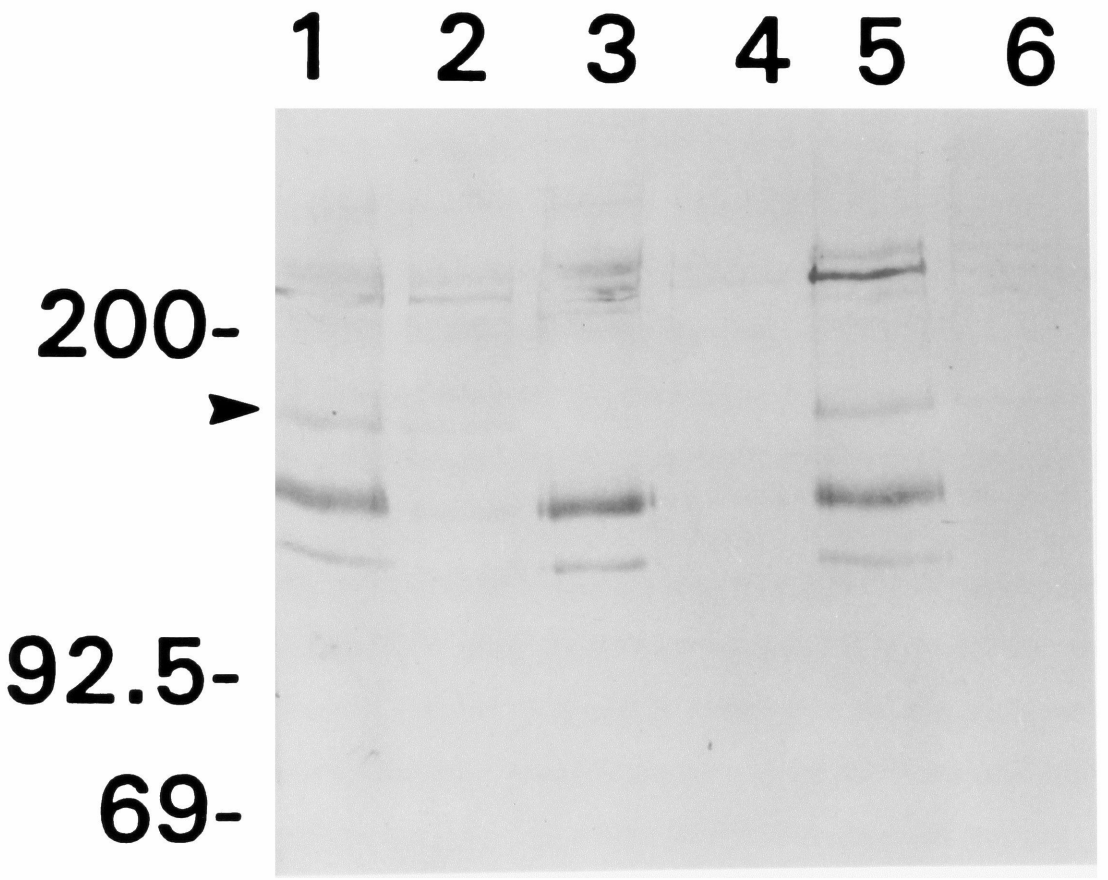
To confirm the specificity of the PDGF receptor tissue staining, Western blot analysis was performed on tissue extracts from two psoriatic patients using antibody PR7212. The band at 180 kd, indicated by the arrow in Figure 28, corresponds to the fully mature PDGF receptor β-subunit (124). The most intensely stained band corresponds to the 164 kd precursor form of the receptor, while the smallest band probably represents a 130 kd PDGF receptor degradation product (124). The bands >200

Figure 27. Staining of normal and growth-activated human skin with a monoclonal antibody to the PDGF receptor by the immunoperoxidase technique. Sections obtained from normal adult human skin (B), chronic skin wounds (D,F), non-lesional psoriatic skin (A,E), and psoriatic lesional tissue (C) were stained with antibody PR7212 directed against the PDGF receptor β chain (A-D,F) and a non-specific isotype control antibody (E). In D, "ep" indicates epidermis not stained by the PDGF receptor antibody. Bar, 40 μ (A-E), 20 μ(F).


A
B
C
D
E
F

kd that were stained with antibody PR7212 were similarly detected with a negative control antibody (data not shown). The immunoblotting study also confirms that psoriatic lesional tissue expresses increased levels of PDGF receptor β-subunit in comparison to an equal quantity of non-lesional psoriatic tissue obtained from the same individual. This finding was true when comparing samples consisting of epidermis with papillary dermal tissue (Figure 28, lanes 1 and 2), reticular dermis samples (Figure 28, lanes 3 and 4), or full-thickness tissue homogenates (Figure 28, lanes 5 and 6). Similar analysis of wound specimens was not possible due to an insufficient supply of biopsy tissue.

Figure 28. Immunoblot analysis of monoclonal antibody to the β-type PDGF receptor. Proteins were separated by SDS-PAGE and then transferred to nitrocellulose. Samples were obtained as described in Chapter 7 from Patients 1 (lanes 1-4) and 2 (lanes 5 and 6). Psoriatic lesional (lanes 1,3,5) and non-lesional (lanes 2,4,6) specimens were prepared from epidermis and papillary dermis (lanes 1 and 2), reticular dermis (lanes 3 and 4), and full-thickness skin (lanes 5 and 6). The arrowhead denotes the full length 180 kd PDGF receptor β chain.


1 2 3 4 5 6
200-
92.5-
69-

4.3 Discussion

The results in this chapter establish that fibroblasts and other dermal cell types in human skin express β-type receptors for PDGF, while epidermal keratinocytes show no detectable expression of this PDGF receptor. Furthermore, the expression of β-type PDGF receptors is increased in the dermis adjacent to chronic skin wounds and in psoriatic dermis, two conditions associated with increased fibroplasia, neovascularization, and epidermal hyperplasia. Since epidermal keratinocytes do not express PDGF α- or β-type receptors (30,118), direct effects of PDGF on human skin are probably limited to dermal cells that express appropriate receptors. As suggested by histochemical staining, dermal fibroblasts and vascular cells may be important targets of PDGF in human skin. High levels of PDGF receptor expression in psoriasis and chronic wound tissue might regulate proliferation, migration, or other functions in dermal cells expressing this receptor (115).

For the PDGF pathway to be biologically important, free PDGF must be available to react with unoccupied PDGF receptors. In acute wounds, PDGF could be provided by platelet degranulation after vascular injury. PDGF release from platelets might not be significant in psoriatic or in chronic wound tissue, since blood vessels are relatively intact. Cultured human keratinocytes synthesize mRNA for PDGF A-chain and produce increased mRNA for PDGF B-chain upon activation with IL-1α or TGF-β (118). Since PDGF receptor β-subunits bind only PDGF B-chains, PDGF released from activated epidermis in psoriasis or adjacent to chronic wounds could contribute to growth of connective tissue elements associated with these conditions. Thus, modulation of epidermal PDGF synthesis and dermal PDGF receptor expression might provide a means for integration of epidermal-dermal functions under different physiological conditions. However, the ability of the PDGF pathway to affect cell proliferation or other functions

may also be dependent on exposure to other growth factors which regulate cell cycle control points distal to that affected directly by PDGF (29).

Fibroblasts cultured from psoriatic plaques have increased mitogenic and chemotactic responses to PDGF (120), suggesting an alteration of the PDGF pathway in this condition that might potentially involve increased PDGF receptor expression as described here. Although psoriatic keratinocytes apparently do not express PDGF receptors (30,118), PDGF might indirectly contribute to epidermal hyperplasia in several ways. Rapidly proliferating epidermis would be expected to have increased nutritional requirements that might be provided by increased PDGF-mediated vascular growth. Smits et al. (125) have recently reported the presence of PDGF receptor β-subunits on rat capillary endothelial cells, though the exact role of these receptors on endothelial cells remains to be elucidated. PDGF stimulates fibroblasts to increase synthesis of IGF-I (116) and IL-6 (126), two factors that can stimulate the proliferation of human keratinocytes (25,127). Furthermore, IGF-I can stimulate keratinocytes to increase EGF receptors, which are increased in epidermis of psoriasis and chronic wounds ((20) and Chapter 2). A similar scheme has been proposed for regulation of epithelial and connective tissue elements in breast carcinomas in which ductal epithelial cells synthesize PDGF that affects both stromal growth and production of IGF-I (117). Epithelial-connective tissue interactions such as these may help to explain how psoriatic dermal fibroblasts increase epithelial growth in some skin equivalent models (128).

Psoriasis and wound repair are two conditions in which growth of multiple cell types is activated. Elucidation of similarities and differences in these two processes may help to decide which features of cell and tissue activation are common to benign hyperplasia and which are characteristic of a particular pattern of growth activation. Psoriasis, in particular, displays a number of unique histological features which may result from actions of many different growth factors, cytokines, and immune elements on skin (20). Common features of psoriasis and chronic wounds are fibroplasia, increased vascular

growth, and epidermal hyperplasia. The apparent ability of dermal cells in both conditions to increase expression of PDGF receptors suggests that common regulatory pathways may control growth of dermal cells in both conditions. PDGF acting via specific receptors may be an important regulator of growth and function of dermal cell types in adult human skin.

Chapter 5: Effects of Anthralin and CSA on the EGF Receptor Pathway in Human Keratinocytes

5.1 Introduction

Current, commonly used anti-psoriatic therapies range from standard first-line topical treatments like corticosteroids, anthralin, and ultraviolet light to systemic therapies like psoralen and ultraviolet A (PUVA), methotrexate, CSA, and retinoids for recalcitrant cases (129). Most of these treatments have been developed through empirical means. For example, in 1925 Goeckerman developed the combined coal tar/ ultraviolet radiation therapy that bears his name as a response to the observation that sunlight improved psoriasis in some patients (130). Similarly, methotrexate, vitamin D-derivatives, and CSA were all first reported to be efficacious for psoriasis in patients receiving these drugs for other conditions (3).

The lack of a rational basis for the efficacy of anti-psoriatic drugs, the toxicity associated with their long-term use, and their limited effectiveness in producing remission of the disease have restricted therapy with these agents. Therefore, the development of more effective, less toxic treatments requires that the mechanism(s) of action of current treatment modalities be clearly established. Since epidermal hyperplasia in psoriasis may be mediated by altered cytokine and/or inflammatory pathways, anti-psoriatic agents may reverse abnormalities in crucial pathways as part of their therapeutic action. Thus, the study of these treatments in the context of their differing abilities to reverse psoriasis-associated changes in growth factor pathways and immune activation could provide insights into the mode of action of these drugs, while also establishing the relative contributions to the psoriatic phenotype of the various abnormalities that have been identified. Most commonly used anti-psoriatic agents appear to have widespread effects on different cell types and may improve psoriasis through combined action on epidermal

and inflammatory cells. This chapter focuses on the effects of two anti-psoriatic drugs, CSA and anthralin, on keratinocyte growth *in vitro* and their effects on the EGF receptor pathway (131,132) in which both the ligand and receptor components of the pathway have been shown to be overexpressed in psoriatic epidermis (26,27,48,72). *In vivo* studies describing the effects of CSA on TGF-α expression in psoriatic epidermis will also be described (133).

CSA and anthralin provide an interesting contrast in psoriatic drug therapies. Although topical anthralin, along with coal tar, is the oldest effective therapy for psoriasis (134) its mechanism of action is far less well studied than systemic treatment with CSA which has been in use for psoriasis only since 1986 (135,136). Additionally, while the initial response to anthralin treatment is relatively slow it frequently results in long-term remission of psoriasis following cessation of therapy. In contrast, CSA treatment generally shows a rapid improvement in psoriasis which also relapses quickly upon the withdrawal of therapy (135,137). These differences may reflect fundamentally different modes of action of these drugs and could even provide clues to identifying preferred targets for obtaining long-term remissions of psoriasis.

Despite the initial fortuitous discovery of its efficacy in psoriasis (138), extensive investigation of CSA as an anti-psoriatic drug was only begun in response to the identification of activated T-cells in psoriatic lesions (11). From this observation, it was proposed that immune activation might induce the psoriatic phenotype and that the disease could therefore be responsive to CSA. The drug has proved to be extremely effective in the treatment of severe psoriasis (135,136,139,140). *In vitro,* CSA has effects on both keratinocytes (141-143) and chronic inflammatory cells (144), so that CSA effects on psoriatic skin could involve either altered immunological or epidermal activation. Some therapeutic efficacy of CSA in psoriasis may be related to its suppression of chronic inflammatory cell functions. CSA directly inhibits proliferation of T lymphocytes by modulating transcription of mRNA for IL-2 and IFN-γ (145-150). CSA also diminishes

the ability of Langerhans cells to present antigen to primed lymphocytes in psoriatic patients (144,151). Both of these effects may serve to limit production of IFN-γ and other cytokines by activated T lymphocytes. A rapid decrease in HLA-DR expression by psoriatic keratinocytes, observed in patients treated with CSA (144), may be related to diminished local production of IFN-γ or to direct effects of CSA on epidermal keratinocytes. CSA also inhibits the proliferation of normal human keratinocytes in culture at drug concentrations near those achieved in psoriatic epidermis, but the mechanism of its effect on keratinocytes is unknown (142,143,152-154).

Anthralin as a treatment for psoriasis has its origins in 1876 when chrysarobin (3-methyl dithranol), the naturally occurring active component of a ringworm therapy, was proven effective in a psoriatic whose condition had been misdiagnosed (155,156). Anthralin (dithranol) was first synthesized in 1916 (157) and, while treatment regimens have changed over the years, the drug has been in use continuously as an anti-psoriatic therapy up to the present. Anthralin treatment has been reported to inhibit granulocyte function, lipoxygenase pathway activity, DNA replication and repair, mitochondrial respiration, and calmodulin activity *in vitro* (134,158-166). *In vivo*, anthralin decreases plasminogen activator levels in plaques, inhibits mitochondrial ATP synthesis, and normalizes keratin expression (134,165-167). However, it is unclear how anthralin treatment affects the growth factor/cytokine pathways which might regulate epidermal hyperplasia in psoriatic plaques.

5.2 Results: CSA

5.2.1 CSA Inhibits Keratinocyte Growth *In Vitro*

Previous studies using keratinocytes from normal adult human skin or transformed lines (142,143) have demonstrated significant inhibition of proliferation by CSA concentrations of 3-5 μg/ml. In contrast, inhibition of lymphocyte proliferation at 100 ng/ml or less of CSA has been reported (144). To establish an appropriate concentration range of CSA to study in the serum-free culture system used in these studies, normal human keratinocyte strains derived from neonatal foreskin were cultured exclusively in KGM medium in the presence and absence of CSA. Keratinocyte growth was measured using both tritiated thymidine incorporation and cell counting experiments. Figure 29 shows the dose-response relationship for inhibition of keratinocyte proliferation over a range of 1-50 μg/ml CSA. Half-maximal inhibition in proliferation was attained at approximately 5 μg/ml of CSA, similar to that observed in other keratinocyte strains (142). The lowest dose of CSA tested (1 μg/ml) produced a 50% inhibition of proliferation of PHA-stimulated human lymphocytes measured in parallel experiments, confirming the need for higher CSA concentrations to inhibit keratinocyte proliferation (data not shown). Keratinocytes treated with up to 10 μg/ml CSA for 2 d were >90% viable, indicating a specific effect of CSA on inhibition of cell proliferation. Diminished viability of keratinocytes was detected at CSA concentrations higher than 10 μg/ml, which appears to be outside the pharmacologic concentration of CSA in human epidermis (143). The ability of CSA to inhibit DNA synthesis was independently confirmed by flow cytofluorometry analysis described below. The ability of CSA to diminish keratinocyte proliferation over several population doublings was assessed by direct cell counting. In rapidly proliferating cells, concentrations of CSA between 1 and 10 μg/ml inhibited

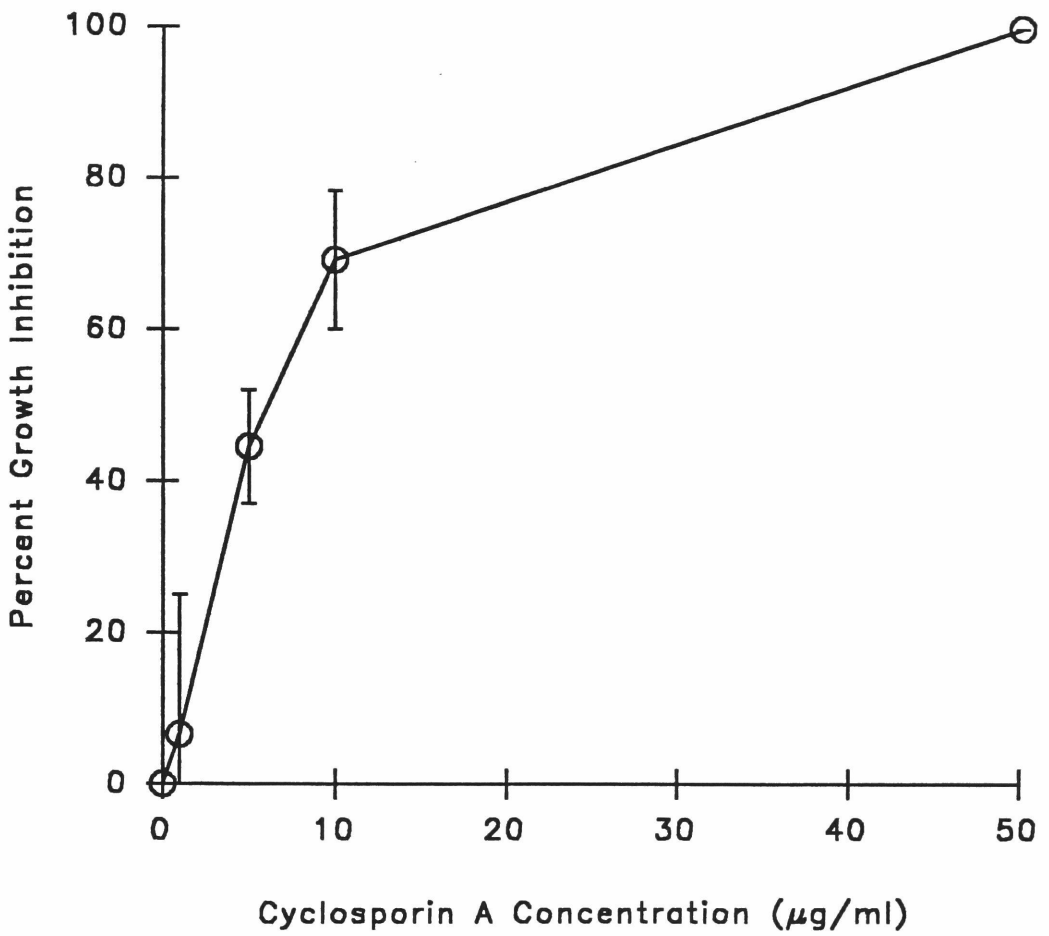


Figure 29. The effect of CSA on keratinocyte proliferation. Inhibition of DNA synthesis by cultured human keratinocytes grown in the presence of CSA or carrier in KGM for 48 h was measured by tritiated thymidine uptake. Results are expressed as percent mean inhibition of triplicate measurements.

proliferation by 42-98% (Table 2).

Table 2. -- Effect of CSA on Keratinocyte Proliferation*

Culture Condition	No. of cells, x 10^3	% Inhibition
Control	1526 ± 113	...
CSA-		
1 μg/ml	972 ± 56	42
5 μg/ml	383 ± 58	87
10 μg/ml	240 ± 10	98

* There were 215,000 keratinocytes present in a 25-cm^2 culture plate at the initiation of the experiment. After 3 d, cell counts were measured using an electronic counter.

5.2.2 CSA Inhibits Keratinocyte Cell Cycle Progression in G_1

To determine the effect of CSA on the keratinocyte cell cycle, flow cytofluorometry was used to assess DNA content of keratinocytes treated for 6, 18, or 24 h with 1-10 μg/ml of CSA. Figure 30 displays the distribution of keratinocytes in G_1 or subsequent phases of the cell cycle (S, G_2, or M). After 18 to 24 h of treatment with CSA, an accumulation of keratinocytes in the G_0/G_1 phase of the cell cycle was detected with CSA concentrations from 1-10 μg/ml, with a concomitant decrease in the S + G_2 + M phases (Figure 30). Separate measurement of S phase in treated keratinocytes showed a 30% reduction in S phase keratinocytes after 24 h of treatment with 5 μg/ml CSA (data not shown). Further characterization of the CSA effect was achieved with simultaneous measurement of DNA and RNA content in cells by flow cytofluorometry that segregates keratinocytes into "A" and "B" populations (168). The A keratinocytes are smaller cells with a more basal appearance that have lower RNA content and less rapid cell cycle progression, whereas B keratinocytes have a larger cell size, more RNA, and a faster cell cycle progression (168). Treatment of keratinocytes with 5 μg/ml CSA for 24 h resulted in a reduction in the B keratinocyte population by 54% (data not shown). These results were confirmed with rapidly proliferating (10% confluent) cultures of keratinocytes (Table 3). Treatment with CSA (5 μg/ml) caused an accumulation of cells in the G_0/G_1 phase of the cell cycle with an accompanying decrease in the proportion of cells in S phase by 63% and G_2/M phases by 51% ($P < 0.001$). Taken together, proliferation and cell cycle data suggest that CSA inhibits keratinocyte proliferation, inducing a G_1 accumulation or block, and shifts the population of proliferating cells toward a slower dividing pool.

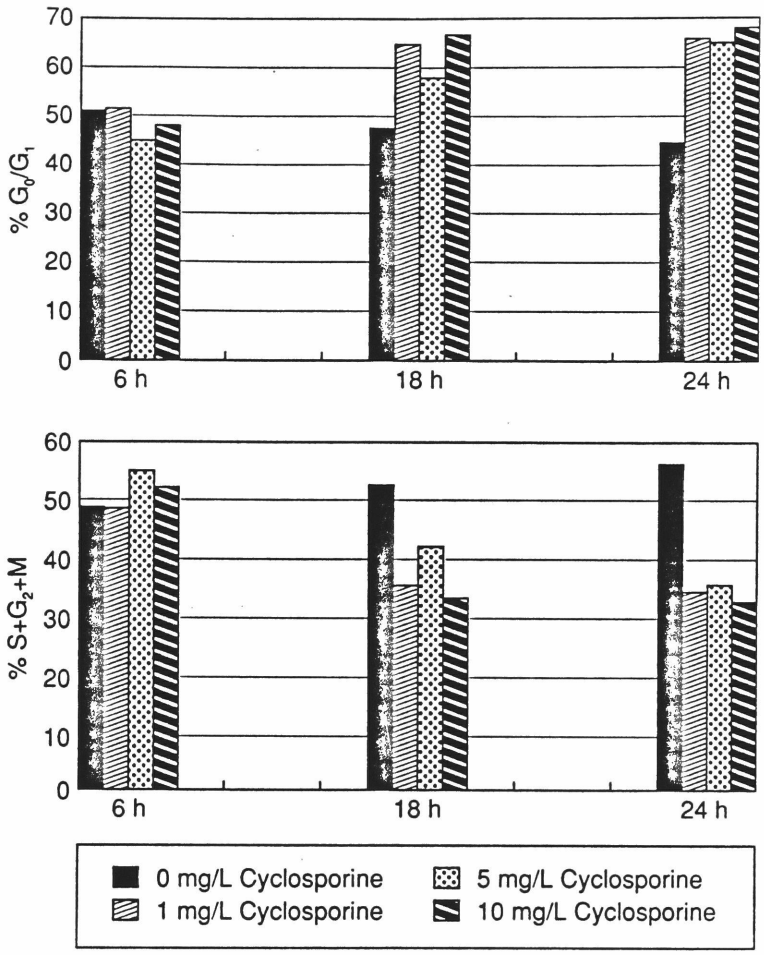


Figure 30. Effect of CSA on keratinocyte cell cycle progression. The effects of CSA on the cell cycle were assessed by flow cytometric measurements of acridine orange-stained keratinocytes. The upper part of the figure represents the percentage of keratinocytes in the G_0/G_1 phase, while the lower part represents the cells in the S + G_2 + M phase.

Table 3. -- Effect of CSA on Keratinocyte Cell Cycle Progression*

Cell Cycle Phase	Proportion of Cells, % Control	CSA, 5 μg/ml	P
G_0/G_1	53.0 ± 2.25	80.4 ± 0.55	<.001
S	28.6 ± 3.2	10.6 ± 0.57	<.001
G_2/M	18.4 ± 0.99	9.0 ± 0.2	<.001

* Keratinocytes were 10% confluent at the initiation of the experiment. Control cultures received the same concentration of DMSO as was present in the CSA-supplemented cultures. Keratinocytes were cultured for 24 h prior to cell cycle analysis.

5.2.3 Effect Of CSA on Keratinocyte Transglutaminase Activity

Activity of transglutaminase, an enzyme synthesized by keratinocytes, increases with terminal differentiation (169). To determine whether the antiproliferative effect of CSA on keratinocytes was associated with induction of terminal differentiation, transglutaminase levels were measured after 2 d with CSA (Table 4). In comparison to control cultures, 5 μg/ml CSA did not increase transglutaminase levels significantly. At 10 μg/ml CSA, an approximately 50% increase in transglutaminase was observed. However, in patients receiving high oral doses of CSA (14 mg/kg/day), cutaneous levels of CSA exceeding 3 μg/ml are not achieved (143). Therefore, the observed *in vitro* rise in transglutaminase levels at 10 μg/ml CSA is of questionable clinical significance. In these experiments, PMA was included as a positive inducer of differentiation (169). Treatment

of keratinocytes with PMA induced a 2.9-fold increase in transglutaminase activity after 2 d of treatment. Parallel experiments demonstrated a concomitant 3-fold increase in cornified envelope production after PMA treatment (data not shown) confirming the utility of using phorbol esters to induce terminal differentiation and of measuring transglutaminase levels as a marker of keratinocyte terminal differentiation.

Table 4. -- Effect of CSA on Keratinocyte Differentiation*

Samples	Tritiated Putrescine, cpm/mg of Protein
Control	1460 ± 304
+ CSA, 5 μg/ml	1502 ± 422
+ CSA, 10 μg/ml	2222 ± 324
+ PMA	4225 ± 411

*Normal keratinocytes were cultured in KGM in the presence of CSA for 2 d. Control cultures received the same concentration of DMSO as was present in the CSA-supplemented cultures. Transglutaminase was then assayed by the incorporation of tritiated putrescine into casein. 100 ng/ml PMA was used as a positive control.

5.2.4 Effect of CSA on TGF-α Expression in Keratinocytes

An important feature of psoriatic epidermis which could be affected by CSA treatment is overexpression of TGF-α mRNA and protein by involved keratinocytes (26,27). The ability of CSA to modulate expression of TGF-α mRNA and protein was assessed in normal keratinocytes treated with up to 5 μg/ml CSA for 1 d. Total RNA was

extracted from control or CSA treated keratinocytes and size-fractionated RNA was probed for TGF-α expression by Northern blot analysis (Figure 31). Even though the increase in TGF-α mRNA in CSA-treated keratinocytes was less than that produced by the known TGF-α inducer PMA (43) (data not shown), a clear dose related increase in TGF-α mRNA was seen in CSA treated keratinocytes. Adjustment for total RNA content as measured by hybridization with an actin-specific probe did not affect these findings (data not shown). Thus, CSA treatment of keratinocytes did not reduce expression of TGF-α mRNA at concentrations that inhibit proliferation and some CSA concentrations even increased TGF-α mRNA, either through effects on transcription or stability of message.

To determine whether CSA might affect translation of TGF-α mRNA into protein or release of mature TGF-α by cells, the amount of TGF-α protein released from keratinocytes treated for 2 d with CSA or PMA was measured with a quantitative radioimmunoassay (91). As shown in Table 5, the quantity of soluble TGF-α released into supernatants of CSA treated cultures was not altered, whereas a 2-fold increase was produced by PMA treatment. Thus CSA appears to have little effect on overall synthesis or release of TGF-α from normal keratinocytes.

Figure 31. CSA increases TGF-α expression in cultured human keratinocytes. Keratinocytes were treated for 24 h with KGM with DMSO (control) or KGM with CSA. Cellular RNA was extracted, separated on an agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized with a TGF-α probe. Lanes 1-6 represent the following: 1- control, 2- 0.01 μg/ml, 3- 0.1 μg/ml, 4- 1 μg/ml, 5- 2.5 μg/ml, and 6- 5 μg/ml CSA. The increase in CSA transcription was not affected when standardized with an actin-specific probe (not shown).

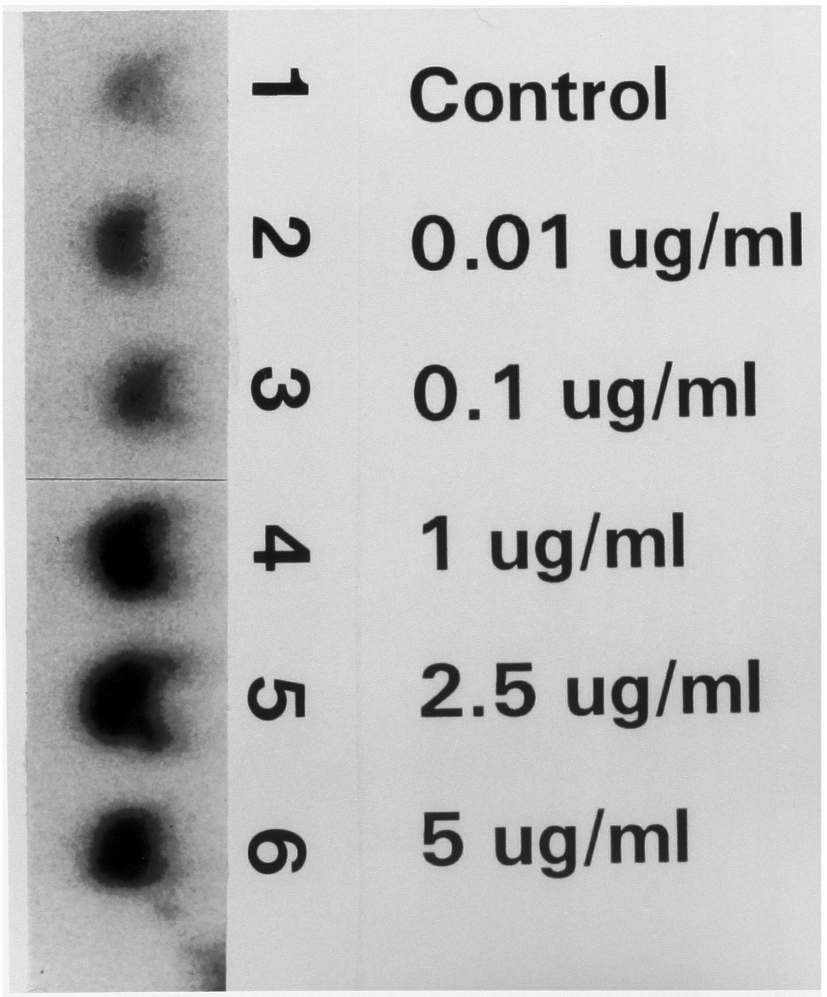

1 Control
2 0.01 ug/ml
3 0.1 ug/ml
4 1 ug/ml
5 2.5 ug/ml
6 5 ug/ml

Table 5. -- Effect of CSA on the Secretion of TGF-α by Cultured Keratinocytes*

Samples	**TGF-α Culture Supernatant, μM**
Control	3.6
+ CSA, 5 μg/ml	3.6
+ PMA	6.4

* Normal confluent keratinocytes were cultured in KGM to keep the cell number fairly constant throughout the experiment. TGF-α concentration in the culture supernatants was measured after 2 d by using a radioimmunoassay kit (Biotope, Redmond, WA). 100 ng/ml PMA was used as a positive control.

5.2.5 Effect of CSA on EGF Receptor Expression in Keratinocytes

Even though CSA did not appear to greatly affect TGF-α expression by keratinocytes, previous studies have suggested that CSA might decrease EGF receptors in keratinocytes (91) that could serve to limit TGF-α-mediated mitogenic signaling in psoriasis. Since keratinocyte proliferative inhibition is well established after 24 h of treatment with 5 μg/ml CSA (Figure 30), EGF receptor numbers were measured after such treatment to assess whether short-term growth inhibition was mediated via the EGF receptor. Subconfluent, rapidly proliferating keratinocytes maintained in KGM were treated for 24 h with 5 μg/ml CSA, and EGF receptor expression was quantitated by binding of ^{125}I-EGF at a variety of ligand concentrations. Figure 32 displays total ^{125}I-EGF binding to CSA-treated and control cultures (insert) and a Scatchard transformation of these binding data. Control keratinocytes showed an average of 1.81×10^5 receptors/cell with a K_d of 0.45 nM, whereas CSA-treated keratinocytes showed 1.76×10^5 receptors/cell with a K_d of 0.5 nM. Similar results were obtained with keratinocytes treated with CSA in media lacking exogenous growth factors (KBM); CSA also failed to significantly affect either EGF receptor number or binding affinity. In several other experiments in which ^{125}I-EGF binding was assessed at a single low concentration of radiolabeled ligand (0.5 to 3 ng/ml), a maximal decrease of 16% in EGF binding was observed. These data are in agreement with differences in EGF binding at low ligand concentrations displayed in Figure 32. Thus, CSA did not appreciably diminish EGF receptor number in keratinocytes under the conditions tested.

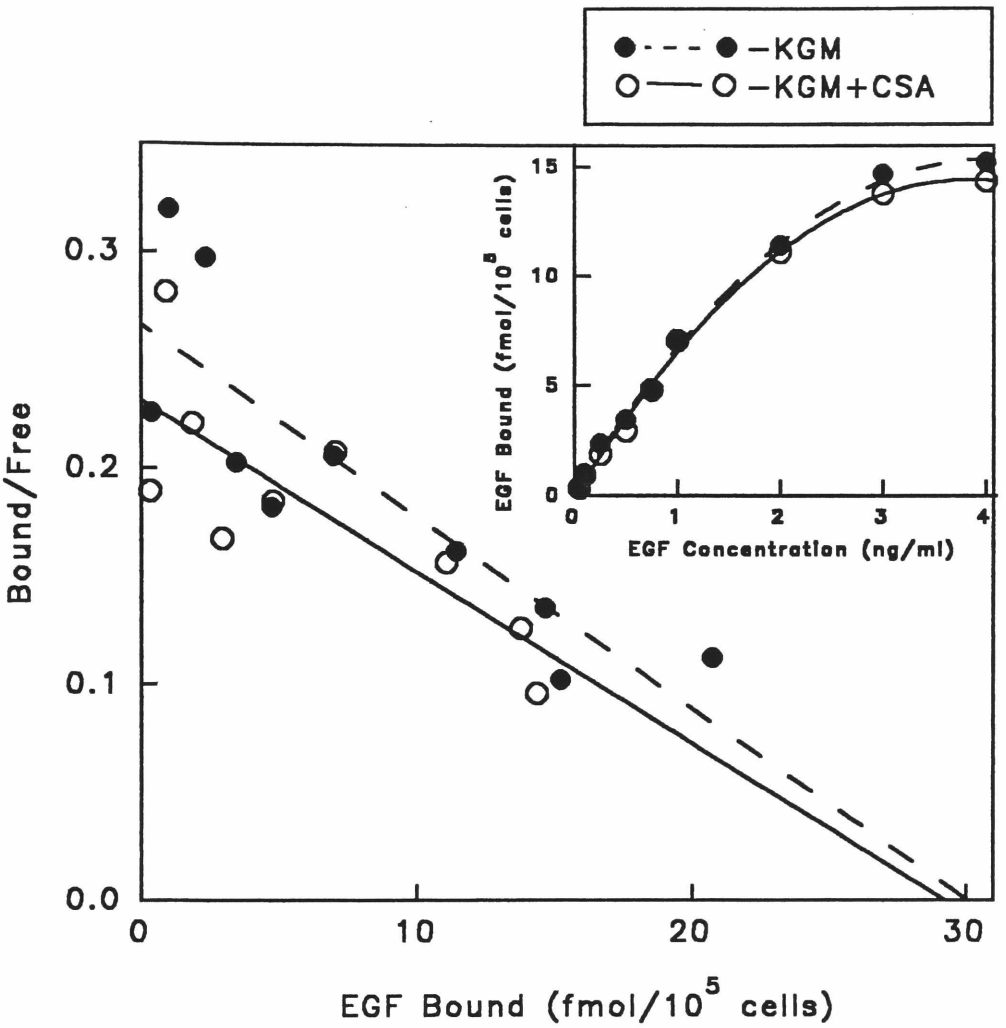


Figure 32. Scatchard analysis of ^{125}I-EGF binding to cultured normal human keratinocytes in response to CSA treatment. Keratinocytes were grown in KGM to 75% confluence and then incubated for 24 h in KGM + DMSO carrier (open circles, solid line) or KBM + 5 μg/ml CSA (closed circles, dashed line). Cells were then processed as described in Chapter 7. Each point represents specific binding of duplicate or triplicate measurements. The insert shows total specific binding to control and treated keratinocytes.

5.2.6 CSA Treatment Does Not Decrease the Expression of TGF-α in Psoriatic Plaques

To determine whether CSA altered TGF-α expression in psoriasis, immunohistochemical and Northern blot analysis of TGF-α was performed on pre- and post-CSA treatment psoriatic plaques (133). Previous immunohistochemical analysis of TGF-α staining in normal skin showed membrane reactivity primarily in the basal keratinocyte layer, while TGF-α membrane staining in psoriasis extended to spinous keratinocytes as well (26). As Figure 33 demonstrates, intense basal and suprabasal membrane TGF-α staining is seen in pre-treatment (Figure 33A) and post-treatment (Figure 33B) psoriatic plaque, while membrane reactivity is limited to the basal layer in uninvolved skin from the same individual. In this individual, TGF-α immunostaining is even more intense in the post-CSA treatment plaque than in the untreated lesional epidermis. High levels of TGF-α immunoreactivity were sustained in all post-CSA treatment psoriatic plaques studied (10/10) with no decrease observed in any lesion.

Figure 34 extends these results to analysis of TGF-α mRNA isolated from the same plaque of the same individual shown in Figure 34. A single 4 kb mRNA species hybridizing with the TGF-α probe was detected in cultured keratinocytes (lane 1) or psoriatic lesional tissue (lanes 2,3) with no significant change in TGF-α or control actin hybridization (lanes 5,6) observed before or after CSA treatment. Thus, the *in vivo* analysis of TGF-α expression in CSA treated patients confirms the *in vitro* finding that CSA does not alter TGF-α protein levels.

Figure 33. Immunoperoxidase staining with the anti-TGF-α specific mAb A1.5 of lesional and non-lesional skin of a psoriasis patient before and after CSA treatment. 130X. Intense membrane staining can be seen throughout the viable layers of involved psoriatic epidermis both before (A) and after CSA treatment (B). In contrast, uninvolved skin from the same patient exhibits intense membrane TGF-α staining only in the basal epidermal layer (C).

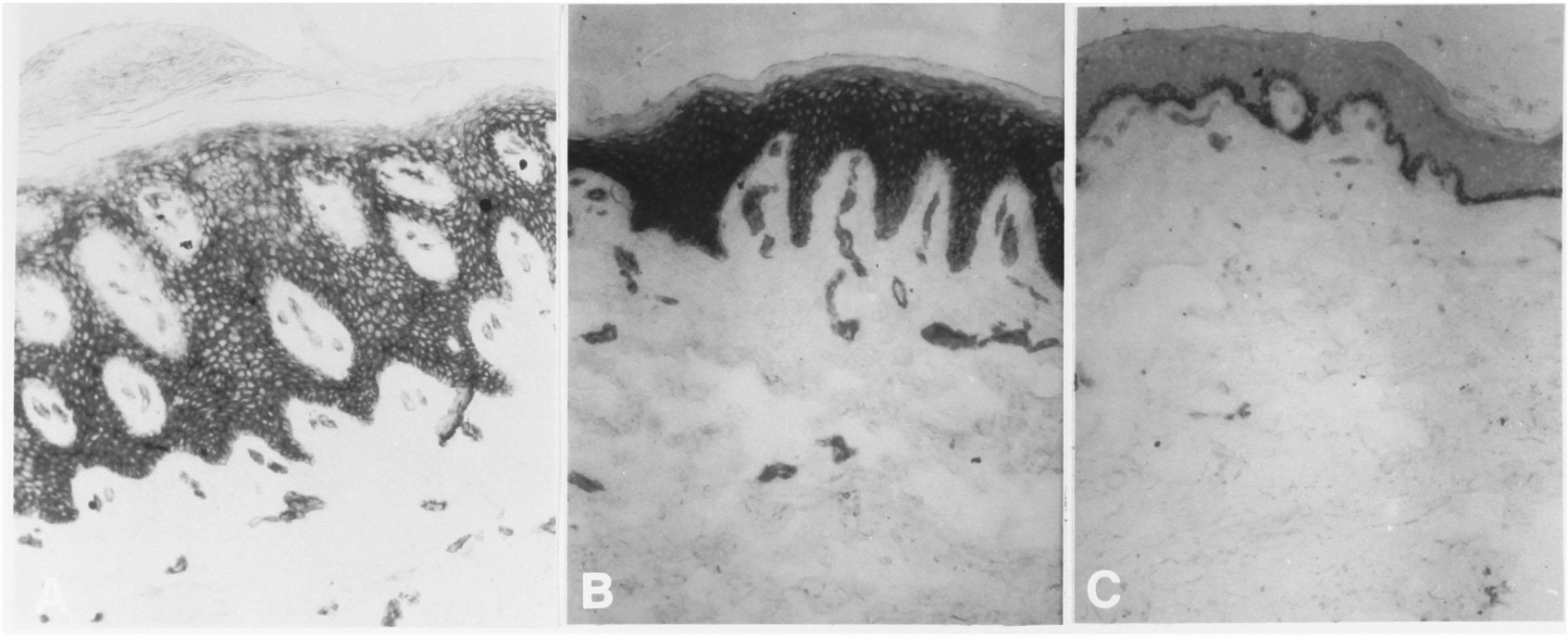
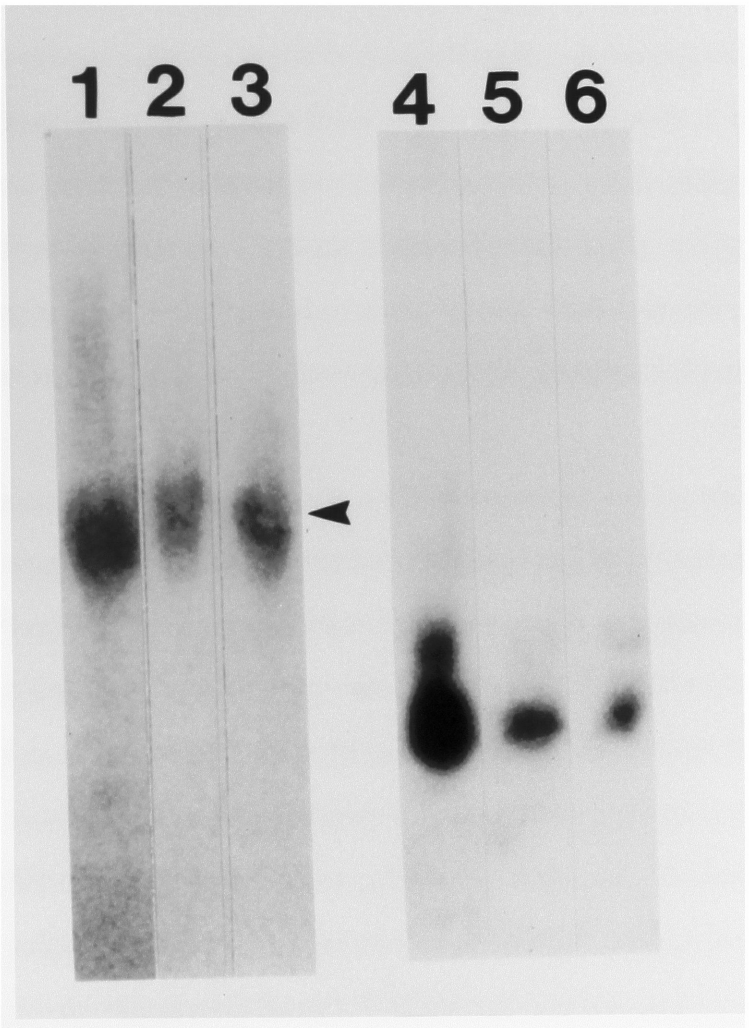

A
B
C

Figure 34. Northern blot analysis of TGF-α transcription in psoriatic plaques before and after CSA treatment. RNA samples from cultured normal human keratinocytes (lanes 1 and 4), pre-treatment psoriatic plaque (lanes 2 and 5), and post-treatment plaque (lanes 3 and 6) were analyzed with cDNA probes specific to TGF-α (lanes 1-3) and actin (lanes 4-6). The arrowhead identifies the 4 kb mRNA transcript for TGF-α.


1 2 3
4 5 6

5.3 Discussion: CSA

Psoriatic plaques display several molecular abnormalities which could be potential targets of therapy. The apparent contributions of multiple growth factors or cytokines to the pathogenesis of psoriasis suggest that therapeutic agents which affect multiple cell growth or inflammatory pathways might be most efficacious in treating this disorder. Furthermore, if psoriasis is viewed as a focus of persistent cell growth or activation, one might predict that agents which block proximal or regulatory points in signal transduction pathways (largely confined to the G_1 phase of the cell cycle) might lead to better responses or longer remission of treated psoriatic lesions. CSA is an interesting therapeutic agent in that it affects the growth of both lymphocytes and keratinocytes (141-144).

CSA appears to block keratinocyte proliferation within the G_1 phase of the cell cycle without inducing terminal differentiation. As suggested by clinical trials, CSA may produce longer lasting remission of psoriasis than agents such as methotrexate which act in the S phase to limit proliferation of growth-activated cells (135,136,139,140,170). The specific mechanism through which CSA decreases keratinocyte proliferation remains unknown. The work described here suggests that an unlikely point of action of CSA is on expression of TGF-α or EGF receptors by cultured keratinocytes. However, keratinocyte proliferation in culture is dependent on expression of multiple, distinct growth factors including EGF, insulin-like growth factors, FGF-related peptides, and several less characterized factors (56). CSA might have suppressive effects on a growth factor pathway independent from that mediated by the EGF receptor or it might affect G_1 progression at a point after the convergence of different growth factor signals.

Clinical studies indicate a rapid decrease in HLA-DR expression by epidermal cells within 1 week of starting CSA therapy (144). IFN-γ is a potent inducer of HLA-DR in

keratinocytes and the rapid change in HLA-DR expression by psoriatic keratinocytes treated with CSA could be due to diminished keratinocyte responsiveness to IFN-γ or to diminished local production by activated T lymphocytes. CSA treated keratinocytes remain responsive to IFN-γ mediated changes, as stimulation of γ-IP-10 gene transcription was observed in response to IFN-γ in keratinocytes exposed to CSA (131). Thus, a primary target of CSA in psoriatic skin may be the T lymphocyte. In fact, lymphocyte proliferation is inhibited by CSA concentrations that are 10 to 100-fold lower than those required to inhibit keratinocyte proliferation (144) and the low doses of CSA currently being used to treat psoriasis may not achieve the concentration necessary to inhibit keratinocyte growth in skin. The ability of CSA to influence HLA-DR expression of epidermal cells and to affect antigen presentation or lymphocyte alloreactivity in psoriatic patients soon after starting therapy further supports a key role for cellular immune activation in the pathogenesis of psoriasis. Recent work using markers of keratinocyte growth activation and immune activation to study psoriatic skin suggests that the primary action of CSA is on decreasing T-cell activation in plaques with accompanying decreases in IFN-γ production, while keratinocyte growth-related changes are resistant to CSA effects (133).

As shown here, epidermal TGF-α expression is among the growth-related changes which CSA does not reverse. Skin biopsy specimens of plaques obtained before and after treatment of psoriatic patients demonstrated unchanged or increased TGF-α levels supporting the *in vitro* data (133). Furthermore, these experiments support the validity of the approach of using *in vitro* experimentation to analyze the effects of anti-psoriatic drugs on cytokine pathways.

5.4 Results: Anthralin

5.4.1 Anthralin Treatment Inhibits Keratinocyte Growth *In Vitro*

Since psoriatic plaques are characterized by both an activated keratinocyte and T lymphocyte compartment (20,171,172), the effects of anthralin on keratinocyte and lymphocyte growth were assessed using cultured normal human keratinocytes and lymphocytes in the presence of varying concentrations of anthralin. These experiments demonstrated that keratinocytes were much more sensitive *in vitro* to the inhibitory effects of anthralin on cell proliferation than were lymphocytes (Figure 35). Anthralin at concentrations of 10 ng/ml to 10 μg/ml was added to actively proliferating foreskin keratinocytes grown under optimal serum-free conditions (Figure 35, upper) and to normal lymphocytes stimulated with the mitogen, PHA (Figure 35, lower). At concentrations of 10 ng/ml, anthralin caused a 98% inhibition of keratinocyte proliferation. In contrast, even at anthralin concentrations of 10 μg/ml, only 50% inhibition of mitogen-induced lymphocyte proliferation was observed. Keratinocyte and lymphocyte viabilities were greater than 99% at concentrations of anthralin less than 10 μg/ml, therefore cell death could not account for marked inhibition of keratinocyte growth by anthralin. These concentrations of anthralin are much less than those achieved in the epidermis of patients treated with 0.1% anthralin *in vivo*. For example, the epidermal level of anthralin that results from the application of a 0.1% preparation is 93.66 μM (134). A 1 μg/ml solution of anthralin is equivalent to a 4.4 μM solution. The concentrations used for the experiments described here are within the range achieved in epidermis after routine clinical use, but the intraepidermal distribution of anthralin after topical administration is unknown.

In order to determine if anthralin inhibited keratinocyte proliferation by

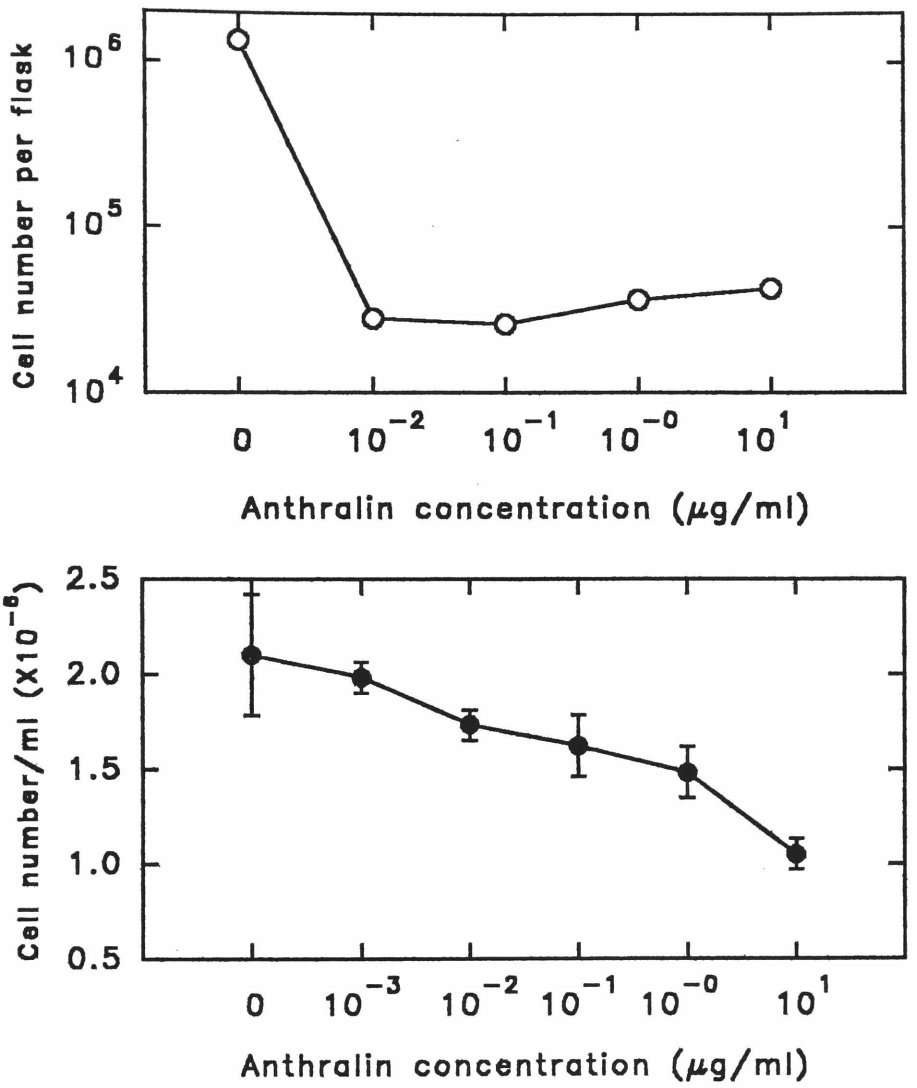


Figure 35. Keratinocytes are more sensitive to the growth inhibitory effects of anthralin than are lymphocytes. Anthralin (0.01-10 μg/ml) was added to normal human foreskin keratinocytes grown in KGM for 5 d (upper) and to PHA-stimulated PBMCs cultured for 4 d (lower). Cell counts were determined using an electronic counter.

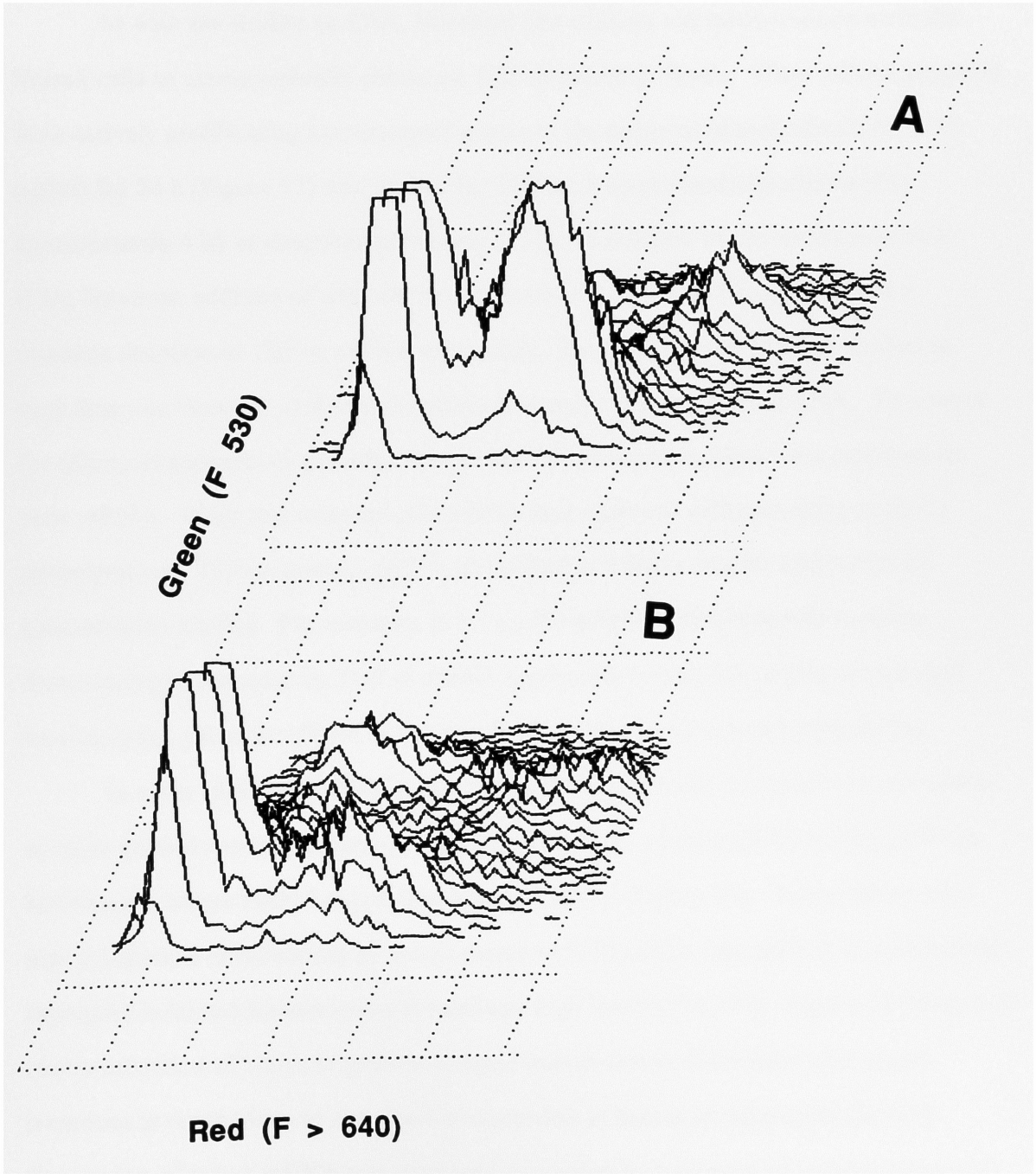
inducing cell cycle specific arrest, anthralin-treated keratinocytes were labeled with acridine orange and then subjected to flow cytometric analysis. Using this technique, both the RNA and DNA contents of individual keratinocytes can be assessed simultaneously. Previous studies have demonstrated that cultured keratinocytes can be divided into 3 populations which differ in their RNA content and proportion of cells which are actively dividing (168,173). Keratinocytes cultured in the presence of anthralin from 0.01 to 1.0 μg/ml showed no significant difference in the percentage of cells in G_0/G_1, S, and G_2+M phases of the cell cycle (Table 6). However, the RNA content of keratinocytes treated with 0.1 μg/ml of anthralin decreased as demonstrated by the reduced "high RNA" peak at 640 nm (Figure 36). This decrease in cellular RNA primarily represents a decrease in ribosomal RNA. In separate experiments, the addition of anthralin did not diminish the accumulation of the rhodamine analog, rhodamine 123 (174), into the mitochondria of viable keratinocytes (data not shown). Since anthralin treatment decreased ribosomal RNA content but did not inhibit mitochondrial-specific dye uptake, anthralin has differential effects on growth-related cellular organelles.

Table 6 -- Anthralin Treatment Does Not Cause Cell Cycle Specific Arrest*

Anthralin (μg/ml)	Cell Cycle Distribution (%)		
	G_0/G_1	S	G_2/M
Control	41.2	40.6	18.2
0.01	37.4	49.4	13.2
0.10	34.3	38.7	27.0
1.0	43.9	41.2	14.9
10.0	44.1	27.1	28.8

*Keratinocytes were 10% confluent at initiation of the experiment. Control cultures received the same concentration of DMSO as was present in the anthralin-supplemented cultures. Keratinocytes were cultured for 24 h prior to cell cycle analysis.

Figure 36. Anthralin decreases total cellular RNA in cultured human keratinocytes. Flow cytometric analysis of acridine orange-stained control (A) and anthralin-treated (0.1 μg/ml) (B) cultured keratinocytes. Fluorescence intensity at 640 nm and at 530 nm is proportional to RNA and DNA content, respectively.


A
B
Green (F 530)
Red (F > 640)

5.4.2 Anthralin Treatment Inhibits Keratinocyte TGF-α Expression *In Vitro*

As with the studies on CSA, Northern blot analysis was performed on anthralin treated cells to assess anthralin effects on TGF-α transcript levels. When mRNA prepared from actively proliferating keratinocytes grown in the presence of anthralin (0.01 to 10 μg/ml) for 24 h (Figure 37) was probed for TGF-α, a single band was observed at approximately 4 kb as described previously (131). In contrast to the results seen with CSA, however, addition of anthralin at concentrations of 0.1 to 10 μg/ml caused a dramatic decrease in TGF-α mRNA expression. The total amount of RNA applied to each lane was identical as shown by ethidium bromide staining of total RNA. To control for effects of anthralin to globally decrease mRNA production, blots were reprobed for actin mRNA. While less actin specific mRNA was observed with increasing anthralin concentrations, TGF-α specific mRNA diminished at a faster rate for each anthralin concentration studied. For example, at 0.1 μg/ml anthralin, densitometric scanning demonstrated a reduction in TGF-α mRNA expression to only 8% of that of untreated keratinocytes, yet actin mRNA remained at 67% of that of untreated keratinocytes.

In order to determine whether anthralin non-specifically decreased the expression of other growth-associated genes, the effect of anthralin addition (0.01 to 10 μg/ml) on keratinocyte c-myc mRNA expression was next tested (Figure 38). Transcription of c-myc is inducible in fibroblasts by serum exposure (175) and c-myc mRNA is constitutively expressed in human keratinocytes at relatively high levels (176,177). Figure 38 (lanes 1-4) shows induction of c-myc in growth-arrested human dermal fibroblasts after serum treatment in comparison to constitutive expression in human keratinocytes (lane 5). Expression of c-myc mRNA was increased somewhat by treatment of keratinocytes with PMA (lane 6) or anthralin (Lanes 7-9). In comparison to expression of TGF-α mRNA or actin mRNA, c-myc mRNA was not diminished by treatment of cells with anthralin

Figure 37. Anthralin inhibits TGF-α mRNA expression in cultured human keratinocytes. Northern analysis was performed under high stringency conditions on actively proliferating keratinocytes grown in the presence of anthralin (0.01 to 10 μg/ml) for 24 h. The total amount of RNA applied to each lane was identical as shown by ethidium bromide staining of total RNA (A). Addition of anthralin at concentrations of 0.1 to 10 μg/ml caused a dramatic decrease in TGF-α mRNA expression (B). The blot was reprobed with an actin specific cDNA probe (C). While less actin specific mRNA was observed with increasing anthralin concentrations, TGF-α specific mRNA diminished at a faster rate for each anthralin concentration studied.

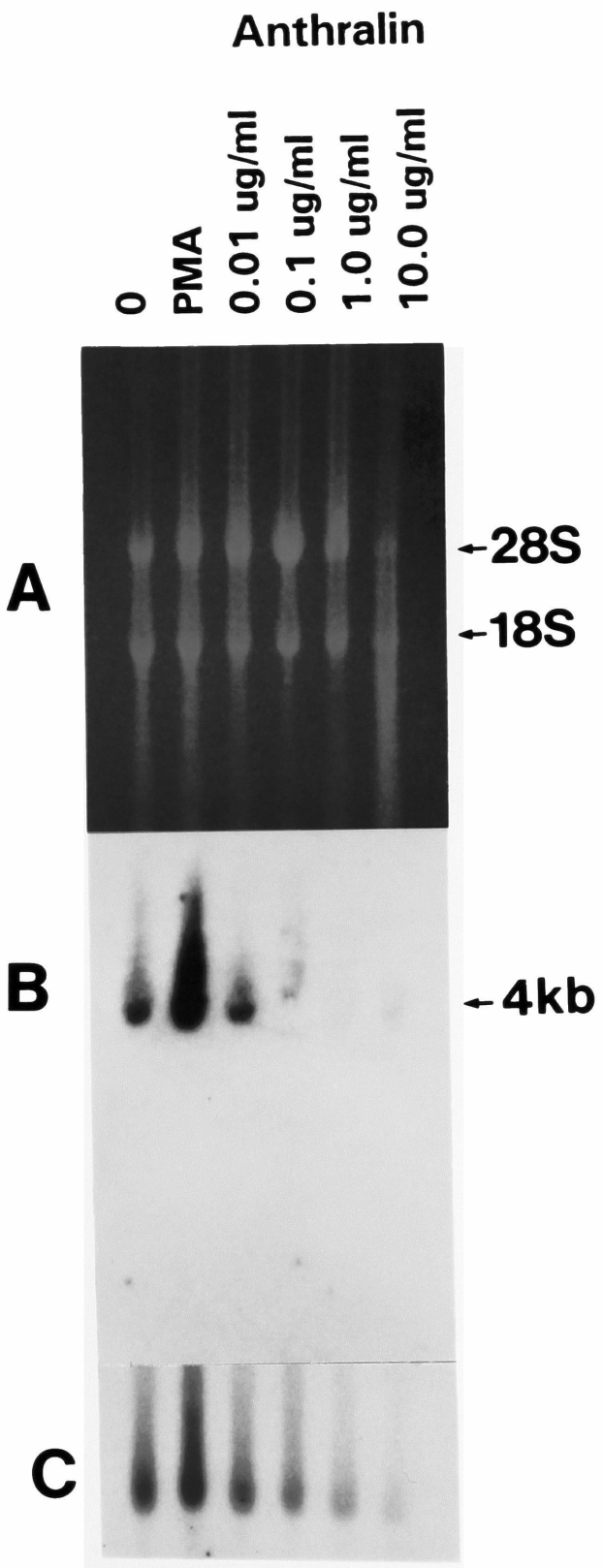
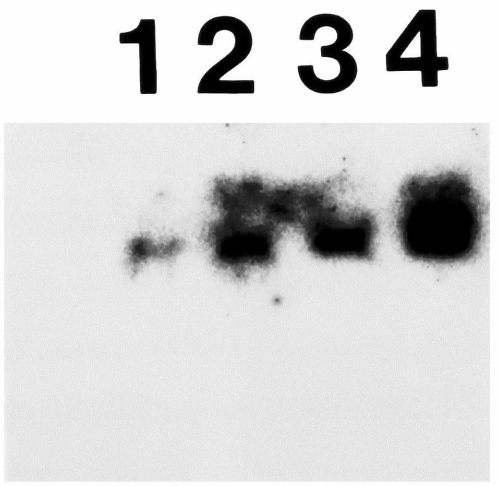

Anthralin
0
PMA
0.01 ug/ml
0.1 ug/ml
1.0 ug/ml
10.0 ug/ml
A
B
C
28S
18S
4kb

Figure 38. Anthralin does not decrease c-myc mRNA expression. Expression of c-myc mRNA is induced in growth arrested human dermal fibroblasts (lane 1), after 10% serum treatment for 0.5 (lane 2), 1.0 (lane 3) and 2.0 (lane 4) hours in comparison to constitutive expression in human keratinocytes (lane 5). Expression of c-myc mRNA was increased somewhat by treatment of keratinocytes with TPA (lane 6) or anthralin at 0.01 (lane 7), 1.0 (lane 8) and 10.0 (lane 9) μg/ml as compared to the control (lane 5).


1 2 3 4


5 6 7 8 9

concentrations as high as 10 μg/ml.

5.4.3 Anthralin Decreases EGF and IGF-I Receptor Binding

As described in Chapter 2, IGF-I and EGF work in synergy to promote keratinocyte proliferation *in vitro*. The effect of anthralin on the binding of EGF and IGF-I to their respective receptors on keratinocytes cultured under optimal conditions, was next investigated (Figure 39). Again in contrast with CSA, addition of 0.1-10 μg/ml anthralin caused a sharp decrease in EGF binding to the EGF receptor. IGF-I binding to the IGF-I receptor was also inhibited but to a lesser extent. The differential sensitivities of the EGF and IGF-I receptors to the effects of anthralin suggests there is specificity to its mode of action and supports the findings of Chapter 2 that these pathways are differentially regulated. In separate experiments, Scatchard analysis revealed that anthralin (1.0 μg/ml) decreased EGF receptor binding affinity and cell surface receptor numbers (Figure 40). The K_d for binding of EGF to its receptor in the absence of anthralin was 0.5 nM, whereas in the presence of anthralin it was 2.1 nM. EGF receptor cell surface numbers were decreased to a lesser extent. The number of receptors per cell was 2.1×10^5 in the absence of anthralin and 1.7×10^5 in the presence of anthralin. Thus, reduced binding of EGF to its receptor appears to be primarily mediated by diminished binding affinity.

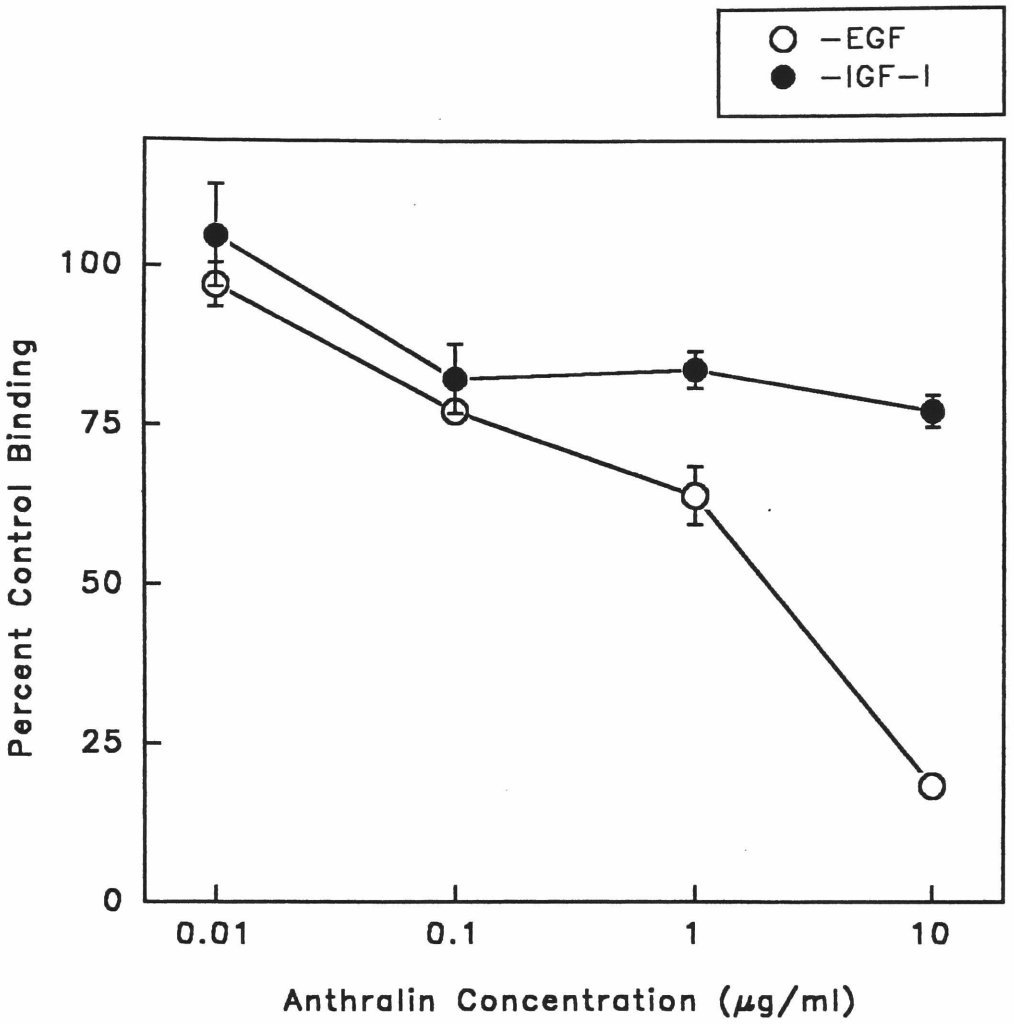


Figure 39. Anthralin decreases EGF receptor and IGF-I receptor binding on cultured human keratinocytes. Keratinocytes grown in KGM were transferred for 24 h to KGM + DMSO carrier (control) or KGM containing 0.1-10 μg/ml anthralin. Anthralin caused a sharp decrease in EGF binding (open circles) to the EGF-receptor, while IGF-I binding (closed circles) to the IGF-I receptor was inhibited to a lesser extent. Values were determined in triplicate and are expressed as the percent mean specific binding to control cells ± standard deviation.

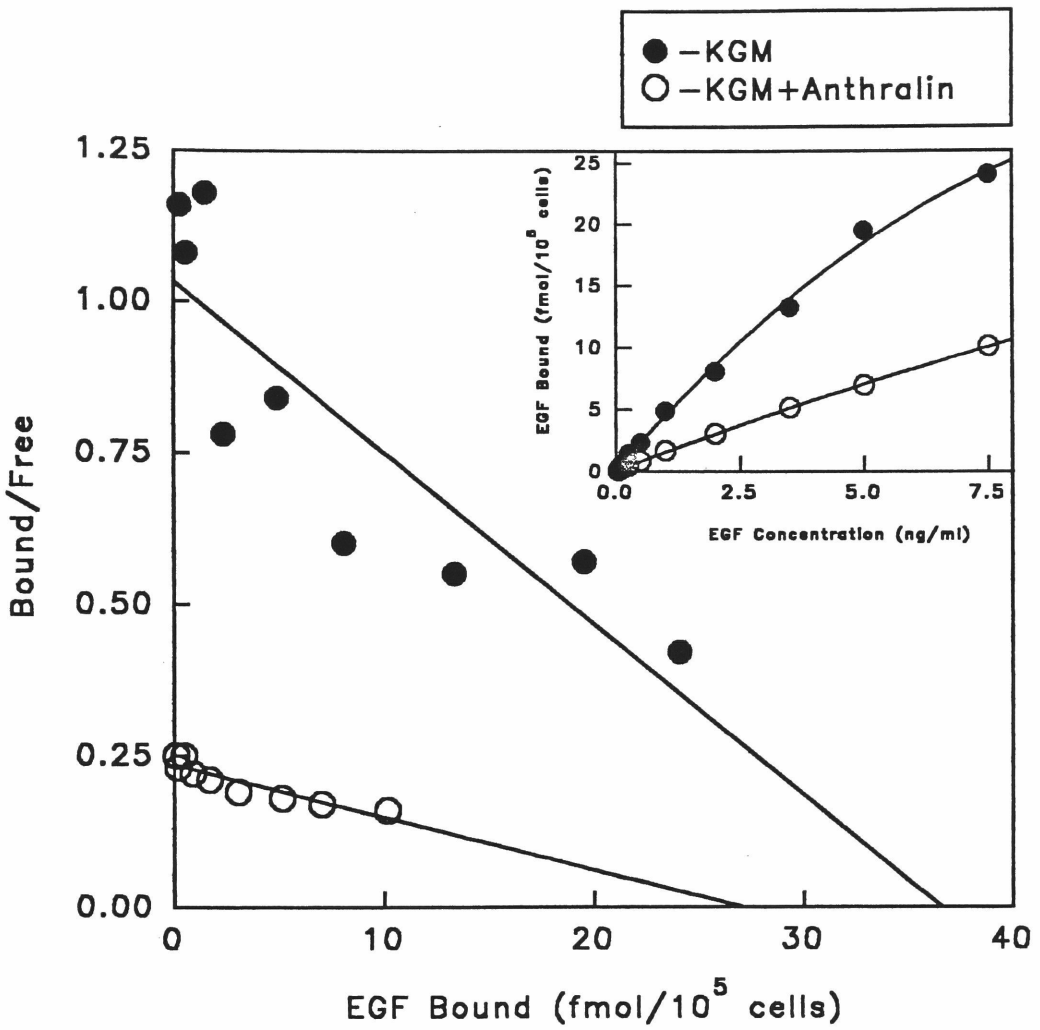


Figure 40. Scatchard analysis of anthralin effects on EGF receptor binding. Keratinocytes were grown in KGM to 75% confluence and then incubated for 24 h in KGM + DMSO carrier (closed circles) or KGM + 1 μg/ml anthralin (open circles). Cells were then processed as described in Chapter 7. Each point represents specific binding of triplicate measurements. The insert shows total specific binding to control and treated keratinocytes. The Scatchard plot indicates that anthralin decreases both EGF-receptor binding affinity and receptor numbers.

5.5 Discussion: Anthralin

This work demonstrates that pharmacologically relevant concentrations of anthralin have the ability to strongly inhibit keratinocyte proliferation, possibly by affecting early steps in mitogenic signaling pathways. Anthralin diminishes ligand binding to the EGF receptor via a reduction in binding affinity. Previous work has demonstrated that EGF receptor affinity and ligand-induced tyrosine kinase activity is greatly reduced by PKC activation as a result of phosphorylation of the EGF receptor on threonine residue 654 (77-80). Anthralin may also block EGF receptor effects by decreasing binding affinity, although the mechanism of action for anthralin may be different from that of PKC.

Anthralin treatment significantly decreases the abundance of mRNA for TGF-α. Since TGF-α mRNA abundance is regulated in keratinocytes by activation of the EGF receptor, the diminished TGF-α in anthralin treated cells could be mediated either via effects of anthralin on the EGF receptor or by direct effects on TGF-α mRNA transcription or stability. Anthralin might also diminish keratinocyte proliferation through its effects on the IGF-I receptor, though the effect of anthralin on this growth factor receptor is quantitatively smaller than its effects on the EGF receptor. Other effects of anthralin on epidermal keratinocytes, including diminished respiration (166), diminished DNA synthesis (163,164), and normalization of keratin expression (165), may be secondary consequences of diminished mitogenic signaling from EGF, IGF-I, or other membrane growth factor receptor systems.

The ability of anthralin to affect EGF receptor expression and TGF-α mRNA in human keratinocytes contrasts markedly with CSA. While CSA also directly inhibits keratinocyte proliferation, it does not significantly diminish EGF receptor expression or TGF-α mRNA production by human keratinocytes. CSA induces a proliferative block in the G_1 phase of the cell cycle, whereas anthralin does not block proliferation at a single

point in the keratinocyte cell cycle. Thus, these anti-psoriatic compounds which have direct epidermal effects, may have different mechanisms of inhibiting keratinocyte proliferation *in vitro*. *In vivo,* the anti-psoriatic mechanism of anthralin and CSA may be significantly different, since lymphocyte proliferation is more strongly suppressed by low concentrations of CSA, whereas keratinocyte proliferation is more strongly inhibited by low concentrations of anthralin.

Clinical treatment of psoriatic lesions with anthralin appears to produce relatively long remissions after cessation of therapy, whereas there is a predictable relapse of active psoriasis shortly after cessation of therapy with CSA (135,137). The ability of anthralin to decrease keratinocyte mitogenic activation by TGF-α might be important in this regard, as CSA has relatively little effect on this pathway *in vitro* or *in vivo* (133,178). In preliminary studies, treatment with 0.1% anthralin *in vivo* decreased immunoreactive TGF-α in a treated psoriatic plaque consistent with *in vitro* results described above. Clearly, further analysis of psoriatic tissue treated *in vivo* with anthralin will be needed to further clarify its mode of action, especially in comparison to other anti-psoriatic agents.

Chapter 6: Concluding Remarks

The results described in this thesis expand upon previous observations of increased expression of EGF receptors and TGF-α in psoriatic epidermis (26,27,48,72). It is now apparent that altered receptor expression of other tyrosine kinase growth factor receptor pathways may contribute to psoriatic epidermal hyperplasia as well. In particular, IGF-I receptor expression may be a critical regulator of epidermal proliferative capacity. Activation of the IGF-I receptor may be necessary for mitogenic stimulation by the EGF or FGF receptor pathways to occur. Furthermore, recent growth studies in our laboratory indicate that another growth promoting cytokine produced by keratinocytes and overexpressed in psoriatic skin, IL-6 (25), also acts synergistically with IGF-I to stimulate keratinocyte growth (H. Uyttendaele, personal communication). Thus, modulation of IGF-I receptor activation may be a critical checkpoint in maintaining normal keratinocyte proliferative regulation.

Cytokines produced by multiple cell types in the skin are likely to form a complex network of interactions that contribute to the psoriatic phenotype. Figure 41 presents a schematic interpretation of how these interactions might enable communication among various compartments of the skin. Work described in Chapter 5 indicates that the β-form of the PDGF receptor is overexpressed in psoriatic blood vessels and in dermal fibroblasts. Cultured keratinocytes constitutively synthesize mRNA for PDGF-A which does not activate the PDGF-β receptor, but mRNA for PDGF-B, which does activate the PDGF-β receptor, can be induced in keratinocytes by treatment with IL-1α or TGF-β (118). Since keratinocytes do not express PDGF receptors, regulation of paracrine release of PDGF by keratinocytes may control growth activation of dermal blood vessels and connective tissue elements. PDGF treatment of cultured fibroblasts induces both IGF-I and IL-6 synthesis (116,126). Since psoriatic fibroblasts overexpress PDGF receptors and exhibit an increased sensitivity to PDGF (120), they may produce increased quantities of IGF-I and

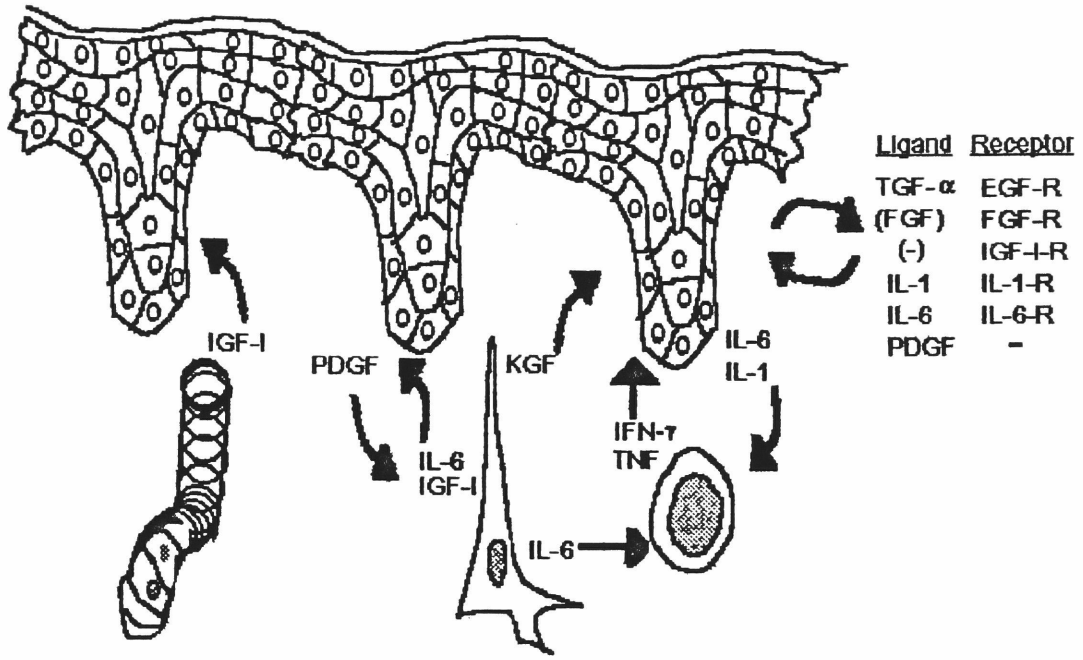


Figure 41. A simplified view of the cytokine network in the skin. Cytokine production by keratinocytes (top), fibroblasts (lower middle), and immune cells (lower right) as well as circulating factors available from leaky capillaries (lower left), could all contribute to psoriatic dermal and epidermal hyperplasia as well as activation of the immune response. Regulation of receptor expression could be critical to the proper cellular targeting of cytokine production. Discussion of how cytokine and receptor abnormalities may contribute to psoriatic hyperplasia is provided in the text.

IL-6. IGF-I, which could also be derived from leaky, fenestrated capillaries in the papillary dermis (93), could in turn stimulate keratinocyte proliferation through increased IGF-I receptors in the epidermis. IGF-I receptor activation may increase EGF receptor levels, perhaps by "unmasking" the pool of "latent" immunologically identifiable EGF receptors which do not bind ligand in normal skin (72). These EGF receptors could in turn be activated by the increased TGF-α found in psoriatic epidermis (26,27). Elevated levels of IL-6, which could be produced by dermal fibroblasts in response to PDGF or by keratinocytes, might act to stimulate keratinocyte proliferation as well as immune activation (25). Additionally, KGF produced by fibroblasts (101,105) or bFGF released from keratinocytes in response to tissue injury (as seen with the Koebner phenomenon) could activate epidermal FGF receptors. Thus, cytokines from dermal fibroblasts, blood vessels and keratinocytes could potentially act to stimulate keratinocyte growth through the common end point of synergistic interactions between activated IGF-I receptors in conjunction with activated IL-6, FGF, and/or EGF receptors. Consequently, targeting of the epidermal IGF-I receptor for anti-psoriatic therapy could possibly diminish the growth promoting activity of multiple cytokine pathways upon psoriatic epithelium.

While this oversimplified model for cytokine involvement in psoriatic epidermal hyperplasia certainly underestimates or omits the function of some cytokines and cell types (particularly T lymphocytes), it does provide a basis for assessing the role of different pathways in the psoriatic lesion. Since psoriatic epidermis and growth-activated skin in wound healing share many common features (8) including overexpression of epidermal EGF receptors and TGF-α (20) as well as dermal PDGF receptors, these features may represent common end responses to growth inducing stimuli. Features unique to psoriasis may be more closely linked to the fundamental pathogenetic defect(s) of the disease. A greater understanding of these common motifs for growth stimulation could provide insights into normal wound healing, hyperplastic diseases like psoriasis, and perhaps even neoplastic diseases like breast carcinoma where similar mechanisms for stromal-epithelial

interactions have been proposed (117).

Even without knowledge of the pathogenesis of psoriasis, it now seems possible to target particular pathways for the rational design of new therapies. These efforts might include one or more of the following approaches: attempts to decrease ligand expression in the epidermis; the use of receptor antagonists, tyrosine kinase inhibitors, or other signal transduction inhibitors; the use of inhibitors of dermal growth activation (particularly targeting the PDGF receptor); treatment with epidermal differentiation inducing agents; and the development of less toxic immunomodulatory drugs. Further studies of the mechanism of action of currently used therapies may provide clues to which cytokine pathway abnormalities should be targeted for treatment. Such studies could eventually lead to the development of rationally designed therapies capable of providing long-lasting remission of psoriatic symptoms with the least amount of toxicity to patients.

Chapter 7: Materials and Methods

Reagents

Reagents were obtained from Sigma Chemical Co. (St. Louis, MO) except where noted.

Chapter 2

Keratinocyte Growth Assay

Normal human keratinocytes were obtained from neonatal foreskins after dermal-epidermal separation was achieved by a 12 h incubation in 1% dispase at 4°C, followed by trypsinization for 30 min at 37°C. Keratinocytes were used for experiments after the second or third passage. Keratinocytes were grown in serum free modified MCDB 153 medium (KGM; Clonetics, San Diego, CA) supplemented with 0.5 μg/ml HC (Clonetics), bovine insulin (5 μg/ml, Clonetics), human recombinant EGF (10 ng/ml, Clonetics), bovine pituitary extract, gentamycin and amphotericin B. Cells were trypsinized and the trypsin was neutralized with DMEM/10% fetal calf serum. Keratinocytes were collected by centrifugation and resuspended in KBM (KBM is KGM without the factors indicated above, Clonetics) containing 0.5 μg/ml HC and antibiotics as a basal growth medium. Factors added to KBM/HC were bovine insulin (5 μg/ml), human recombinant insulin (5 μg/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 (Coulter Electronics, Inc., Hialeah, FL). Cells were photographed 1 and 6 d after seeding with an inverted phase-contrast microscope on Kodak Tmax 100 film (Eastman Kodak Co., Rochester, NY) using a 10X objective.

Immunohistochemistry

Immunoperoxidase staining of cryostat sections of skin from neonatal foreskin, normal adult human skin, and 18 psoriatic patients was performed with mouse monoclonal antibody αIR-3 as described previously (26) using 3-amino-9-ethylcarbazole as the developing reagent. A well characterized mouse monoclonal antibody, αIR-3, specifically recognizing the IGF-I receptor (73) 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 (72). Both antibodies were used at an IgG concentration of 1 μg/ml and staining was compared with an IgG isotypic control.

Receptor Binding Assays

Cultured human neonatal foreskin keratinocytes were grown in KGM to approximately 75% confluence in 24-well tissue culture plates. For experiments performed in KGM, compounds were added in KGM for the indicated times and the plates were then washed 2 x 90 min at 37°C with KBM prior to the assay. For analysis of receptor binding in KBM, keratinocytes grown in KGM were transferred to KBM for 1 d prior to the addition of compounds in KBM. Added compounds were 10 ng/ml PMA (LC Services Corp.; Woburn, MA), 10 ng/ml 4α-PMA (LC Services Corp.), 1.15 mM $CaCl_2$, and the calcium ionophores ionomycin (1 μM) and A23187 (0.1 μM). For transmodulation experiments, cells were transferred to KBM for 1 d, 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 placed on ice and washed once with cold binding buffer (BB: KBM + 5 mg/ml BSA + 20 mM HEPES (pH 7.3)) followed by the addition of 0.25 ml/well of ^{125}I-EGF (Amersham Corp., specific activity 100 μCi/μg) or ^{125}I-IGF-I (Amersham Corp.; specific activity 2000 Ci/mmol) in BB to each well. ^{125}I-EGF or ^{125}I-IGF-I was added at 1 ng/ml, except for Scatchard analysis where the radioisotope was added up to a maximum of 5 ng/ml and supplemented with unlabelled ligand at higher concentrations. In the transmodulation experiments, the unlabelled ligand added was receptor grade EGF (Collaborative Research, Inc., Bedford, MA). In other experiments, the unlabelled ligand added was human recombinant EGF or human recombinant IGF-I (Pepro Tech Corp.; Rocky Hill, NJ). 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% TX-100 and counts were read in a gamma counter. Nonspecific binding was measured with a 1,000-fold excess of unlabelled EGF in the transmodulation experiments and did not exceed 5% of total binding. Nonspecific binding was measured with a 5,000-fold excess of unlabelled EGF or IGF-I in all other experiments and did not exceed 20% 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.

Receptor binding inhibition experiments were performed as described above with 1 ng/ml ^{125}I-IGF-I added with unlabelled IGF-I (Pepro Tech Corp.), insulin (Clonetics Corp.), or mAb αIR-3. Each value represents mean specific binding of triplicate measurements.

Localization of ^{125}I-IGF-I Binding by Autoradiography

The experiment was performed essentially as described for localization of EGF receptor binding sites in human skin (48). Full-thickness excisional biopsy specimens from

normal human skin were sliced into small pieces of approximately 1 mm^2. The samples were then incubated with shaking at room temperature for 90 min in BB (described above) containing 5 ng/ml of ^{125}I-IGF-I or an equal quantity of radioligand in the presence of a 5,000-fold excess of human recombinant IGF-I (Pepro Tech Corp.). The samples were washed 6 x 10 min with BB at room temperature and then fixed overnight in 10% formalin. Samples were then embedded in paraffin and processed for sectioning. Sections (approximately 6 μm) were dipped in 1:1 NTB-2 (Eastman Kodak Co.) emulsion and distilled water. After exposure at 4°C for 8 weeks, the slides were developed 1 min in Kodak D-19 developer at 15°C. Sections were lightly stained with hematoxylin and eosin and mounted in Permount.

Tyrosine Kinase Assays

IGF-I receptor. Split-thickness skin biopsies from 3 psoriatic patients were homogenized in 0.5 ml of lysis buffer (LB: 20 mM Tris (pH 8.0) containing 150 mM NaCl, 10% glycerol, 1% TX-100, 1 mM PMSF, 0.15 U/ml aprotinin, and 1 mM $NaVO_4$) using a motor driven Teflon pestle. After homogenization, insoluble material was removed by centrifugation in a microfuge for 15 min at 4°C. Protein concentration was determined using the Bio-Rad protein assay kit according to the manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA) and 60 μg of each protein sample was immunoprecipitated with 1 μl αIR-3 at 4°C overnight. Subconfluent cultured neonatal keratinocytes grown in KGM were treated for 24 h with KBM prior to lysis. Thirty min prior to lysis, the medium was changed to KBM alone or containing 100 ng/ml IGF-I and reincubated at 37°C. Cultured keratinocytes were then lysed in 1 ml LB/plate and approximately 300 μg protein was immunoprecipitated with 2 μl αIR-3 overnight. All immunoprecipitates were then bound for 4 h to Protein A-Sepharose beads preincubated with rabbit anti-mouse IgG (Organon Teknika-Cappel; Malvern, PA). Immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl in 20 mM

Tris (pH 8.0), and once with kinase buffer (50 mM Tris (pH 7.4), 10 mM $MnCl_2$). The beads were resuspended in 40 µl kinase buffer and the kinase reaction was allowed to proceed for 15 min at room temperature following the addition of 1 µl γ-32P-ATP (Amersham Corp.; specific activity 6000 Ci/mmol). The reaction mixtures were washed twice with 1 ml kinase buffer and then boiled 5 min in 50 µl 2X Laemmli buffer (179). Proteins were electrophoresed by SDS-PAGE using the Laemmli method (179) along with rainbow molecular weight markers (Amersham Corp.) in a 7.5% polyacrylamide gel. Gels were fixed, dried, and exposed at -70°C using XAR-5 film (Eastman Kodak Co., Rochester, NY) and Cronex intensifying screens (DuPont Co., Wilmington, DE).

EGF Receptor. Subconfluent cultured neonatal keratinocytes grown in KGM in 100 mm^2 plates were treated for up to 20 min with either 100 ng/ml PMA or 1.15 mM $CaCl_2$/10 µM A23187. Plates were then transferred to ice and washed once with ice-cold PBS. Keratinocytes were lysed in 10 mM Tris (pH 7.5), 1% TX-100, 10 mM EDTA, 10% glycerol, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µM $NaVO_4$, and 1 mM DTT. Samples were then immunoprecipitated for 30 min at 4°C with 1.5 µl/plate of a rabbit antibody directed against the carboxy-terminal portion of the EGF receptor (180) (a generous gift of Dr. Stuart Decker). Immunoprecipitates were bound to Protein A-Sepharose beads for 30 min and then washed twice with lysis buffer, once with kinase buffer (20 mM HEPES (pH 7.3), 1 mM $MnCl_2$ 1 mM DTT), and then taken up in 25 µl kinase buffer. Kinase reactions were initiated by addition of 1 µl γ-^{32}P-ATP (Amersham Corp.; specific activity 6000 Ci/mmol), allowed to proceed for 5 min at 25°C and terminated by the addition of 5X Laemmli buffer. Proteins were electrophoresed in a 7% polyacrylamide gel as described above.

EGF Receptor Half-Life Determination

Subconfluent cultured neonatal keratinocytes grown in KGM in 100 mm^2 plates

were transferred to KBM for 10 h. The medium was then changed to methionine-free KBM (Clonetics) for 2 h and the cells were then labeled for 12 h in this medium containing 625 μCi/plate of ^{35}S-methionine (Tran^{35}S-label, specific activity 1000 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, CA). At t=0 h, plates were washed twice with KBM and then reincubated in KBM alone or KBM containing 5 μg/ml bovine insulin (Clonetics) for 0, 12, 24, or 48 h. Keratinocytes were then lysed as described above for EGF receptor kinase assays and equal counts of each sample were immunoprecipitated as described above for EGF receptor kinase assays. Immunoprecipitates were washed (180) and samples were electrophoresed as described above. After fixation, gels were treated for 1 h with EN^{3}HANCE (New England Nuclear, Boston, MA), and then processed as described above.

Chapter 3

Materials

Human recombinant aFGF, bFGF, and KGF were obtained from Dr. Robert Goldman, Pepro Tech Corp.. In some experiments, recombinant bFGF from Synergen or Calbiochem (La Jolla, CA) was used as indicated. ^{125}I-bFGF and ^{125}I-KGF were generously provided by Dr. Dennis Fujii, Sterling Drug Inc., Malvern, PA. Rabbit anti-FGF receptor antibody was a generous gift of Dr. Raymond Birge, Rockefeller University.

Keratinocyte Growth Assay

The growth assay was performed as described for Chapter 2 using KBM/HC as basal medium with addition of 100 ng/ml IGF-I (Pepro Tech Corp.) and/or 50 ng/ml bFGF (Synergen).

FGF Receptor Crosslinking

Subconfluent cultured neonatal keratinocytes grown in KGM in 100 mm^2 plates were washed twice with Crosslinking Buffer (CB): ice-cold KBM containing 0.2% gelatin and 20 mM HEPES (pH 7.3). Plates were then incubated for 2 h at 4°C with 1 ml/plate of CB containing 10 ng/ml (8×10^5 cpm total) of ^{125}I-bFGF. Plates containing excess unlabelled human recombinant bFGF had a 500-fold excess of Pepro Tech Corp. bFGF, 5,000-fold excess of Synergen bFGF, or 5,000-fold excess of Calbiochem bFGF. Plates were then washed once with CB, once with CB containing 0.2% BSA, and once with PBS. Crosslinking was then performed by addition of 1 mM disuccinimidyl suberate (Boehringer Mannheim, Indianapolis, IN) in PBS at 4°C for 20 min. Plates were then washed once with 10 mM HEPES (pH 7.3) containing 2 mM EDTA and 0.2 M glycine and once with PBS. Cells were then scraped into Eppendorf tubes and centrifuged briefly in a Beckman microfuge. The pellet was lysed in 50 µl LB on ice for 15 min and centrifuged at 4°C for 10 min. 5X Laemmli buffer was then added to the supernatant, samples were boiled, and one-fifth of each sample was electrophoresed in a 7.5% polyacrylamide gel as described above. Gels were fixed, dried, and exposed as described above.

Immunoblotting

Biopsy specimens from a psoriatic patient were obtained as described in Chapter 2 under IGF-I Receptor Kinase Assays. Equal quantities of protein were electrophoresed in a 7% SDS-PAGE gel and transferred to 0.2 µm nitrocellulose (Schleicher & Schuell, Keene, NH) (181). The nitrocellulose was blocked in a solution of TBST (10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween 20), DET (0.5% sodium deoxycholate, 0.2% NP-40, 0.02% SDS), and CASEIN (5% milk powder, 2 mM Tris (pH 8.0), 0.02% NaN_3, 0.2 mM EDTA) for 1 h and then incubated overnight at room temperature with a 1:2500

dilution of rabbit anti-FGF receptor antibody in TBST/CASEIN. After washing the filter 3 x 10 min in TBST/DET and 3 x 10 min in TBST/CASEIN, a 1:1000 dilution of an alkaline phosphatase conjugated goat anti-rabbit antibody (Tago, Inc., Burlingame, CA) was added for 2 h at room temperature. After further washes in TBST, the blot was developed in 0.2 M Tris (pH 9.5), 10 mM $MgCl_2$, 1 mg/ml nitro blue tetrazolium, and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate.

Receptor binding assays

Cultured human neonatal foreskin keratinocytes were grown in KGM to approximately 75% confluence in 24-well tissue culture plates. The plates were washed 2 h at 37°C with binding buffer (KBM containing 0.15% gelatin and 25 mM HEPES (pH 7.3)) prior to the assay. Plates were placed on ice and washed twice with cold PBS and 0.25 ml/well of a 1 ng/ml solution of ^{125}I-bFGF or ^{125}I-KGF in binding buffer was added. Unlabelled ligand added was human recombinant aFGF, bFGF, or KGF. Plates were transferred to a rocking incubator at 4°C for 2 h and then washed 4 times with cold PBS. Cells were lysed in 0.1 M NaOH/0.1% TX-100 and counts were read in a gamma counter. Values given are mean specific binding of triplicate measurements and are representative of results obtained from at least two different primary cell lines.

Immunohistochemistry

Immunohistochemical staining of cryostat sections of skin from 1 psoriatic patient was performed as described in Chapter 2 using a 1:500 dilution of the rabbit anti-FGF receptor antibody.

Chapter 4

Materials

Monoclonal antibodies to the α-form of the PDGF receptor (292.18) and the β-form of the PDGF receptor (PR7212) (124) were generously provided by Dr. Charles Hart (ZymoGenetics, Inc., Seattle, WA) as was PDGF-BB homodimer.

Receptor Down-regulation Experiment

Cultured human neonatal foreskin keratinocytes and fibroblasts were grown in KGM and DMEM + 10% fetal calf serum respectively in 24-well tissue culture plates to approximately 90% confluence. Fresh medium containing 50 ng/ml PDGF-BB homodimer was added for the indicated times. The plates were washed once with ice-cold binding buffer (KBM or DMEM + 5 mg/ml BSA + 20 mM HEPES buffer (pH 7.3)), approximately 80,000 cpm/well of radioiodinated anti-PDGFR antibodies 292.18 or PR7212 were added in 0.5 ml binding buffer, and the plates were transferred to a rocking incubator at 4°C for 5 h. After 4 washes with binding buffer, cells were lysed in 0.1 M NaOH/0.1% TX-100 and counts were read in a gamma counter. Each condition was tested with duplicate samples. Results are presented as total cpm bound.

Immunohistochemistry

Immunohistochemical staining of cryostat sections of skin from 10 psoriatic patients and 15 chronic wound patients was performed as described in Chapter 2. Mouse monoclonal antibody PR7212 which specifically recognizes the β chain of the PDGF receptor (124) was used at an IgG concentration of 2-5 μg/ml and staining was compared with an IgG isotypic control.

Immunoblotting

Biopsy specimens from two psoriatic patients were homogenized in 0.5 ml of 80 mM Tris (pH 6.8), 1% TX-100, 4 M urea, 2% β-mercaptoethanol, and 10% glycerol

using a motor driven Teflon pestle. Skin from Patient 1 was sampled in two stages; first as a superficial shave biopsy to remove epidermis and papillary dermis followed by a punch biopsy to obtain reticular dermis tissue. The specimen from Patient 2 was biopsied as a full thickness punch biopsy. After homogenization, insoluble material was removed by centrifugation in a microfuge for 15 min at 4°C. Protein concentration was determined by the bicinchoninic acid method according to the manufacturer's instructions (Pierce, Rockford, IL) and equal quantities of protein were electrophoresed in a 7% SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted as described in Chapter 3 using a 1:500 dilution of PR7212 as primary antibody and a 1:1000 dilution of an alkaline phosphatase conjugated goat anti-mouse antibody (Tago, Inc.) as secondary antibody.

Chapter 5

Cell Proliferation Assay

Keratinocytes were grown in KGM as described under Chapter 2. CSA was obtained from Sandoz Corporation (East Hanover, NJ). The powder was diluted in DMSO to a stock concentration of 3 mg/ml. The stock was diluted in culture medium to prepare various concentrations of CSA. Anthralin (dithranol, 1,8-dihyroxy-9[1OH]-anthracenone) was diluted in DMSO to a stock concentration of 10 mg/ml. The stock was diluted in DMSO to prepare various concentrations of anthralin. Control cultures received appropriate dilutions of DMSO alone.

Keratinocytes were cultured in 24 well polystyrene culture plates (Corning) at a density of 2×10^5 cells/well in KGM. The keratinocytes were incubated with various concentrations of CSA or vehicle alone for 48 h. The cells were pulsed with 2 μCi of tritiated thymidine (ICN Biomedicals, Inc.) for the last 4 h, harvested manually after

treatment with cold 10% trichloroacetic acid and 1% SDS, and then counted in a beta scintillation counter. The results are expressed as percent inhibition which is the percentage of ^{3}H-thymidine incorporated in the CSA treated over control keratinocytes. For CSA cell counting experiments, keratinocyte cultures at a density of 1 x 10^4 cells/cm^2 (10% confluency) were incubated for 3 d. For anthralin experiments, keratinocytes were cultured at a density of 5-10 x 10^4 cells/25 cm^2 flask in KGM. The keratinocytes were incubated with various concentrations of anthralin or vehicle alone for 5 d. Cells were counted in a Coulter Counter. Cell viability was assessed by trypan blue dye exclusion.

PBMCs were isolated from heparinized human blood by Ficoll density gradient centrifugation as previously described (182). Various concentrations of anthralin or vehicle were added to PBMCs cultured at a concentration of 1 x 10^6/ml for 4 d in 10 ml RPMI 1640-10% fetal bovine serum plus glutamine and penicillin/streptomycin in the presence and absence of PHA (Wellcome Diagnostics, Temple Hill Dartford, England) at a 1:250 dilution of the stock solution. After 4 d, cell counts were measured using a Coulter Counter. Cell viability was assessed by trypan blue dye exclusion.

Normal human neonatal foreskin fibroblasts were grown in the presence or absence of fetal bovine serum as previously described (20,183).

Flow Cytometry

Keratinocytes used for cell cycle analysis were grown in serum-free media and then cell cycle distribution was determined using flow cytometric analysis of acridine-orange-treated keratinocytes as previously described (168). Aliquots of 0.2 ml containing 1-4 x 10^5 cells in minimal essential medium plus fetal bovine serum were mixed with 0.4 ml of 0.08 N HCl, 0.15 N NaCl, and 0.1% TX-100 at 4°C. Cells were stained 30 seconds later by the addition of 1.2 ml of a solution containing 0.15 N Na_2HPO_4, 0.1 M citric acid buffer (pH 6), 1 mM disodium EDTA, 0.15 N NaCl, and 6 μg/ml acridine orange (Polysciences, Warrington, PA). Under these conditions, interactions of the dye with

DNA resulted in green fluorescence with a maximum emission of 530 nm (F530), whereas interaction of the dye with RNA gave red metachromasia at 640 nm (F640). The intensities of these reactions are proportional to the DNA and RNA content respectively. Specificity of the staining was evaluated by treatment of the cells with RNase A (Worthington Biochemical Corp., Freehold, NJ) or with DNase I. Fluorescence of individual cells was measured in an Epics 752 Cell Sorter Coulter Cytometer. The red and green emissions from each cell were separated optically and the integrated values of the pulses were quantitated by separate photomultipliers. Background fluorescence was automatically subtracted; 1 x 10^4 cells were counted per sample.

Transglutaminase Assay

Keratinocyte transglutaminase activity was assayed by an adaptation of the method of Yuspa et al. (169). Cells from 4 confluent plates were scraped and washed with PBS and then with assay buffer (50 mM Tris (pH 7.5), 0.13 M NaCl and 8.3 mM $CaCl_2$). The cells were suspended in 225 µl of buffer and sonicated to prepare the cell lysate. The reaction mixture consisted of 100 µl cell lysate, 50 µl of casein (1.2 mg) and 50 µl of ^{3}H-putrescine (New England Nuclear, specific activity 17.9 Ci/mmol). ^{3}H-putrescine was added to cold putrescine to a total concentration of 25 mM. After 30 min at 37°C, 50 µl of reaction mixture was spotted on Whatman 3 MM filter paper strips and immediately immersed in cold 10% trichloroacetic acid. The filter papers were agitated through three 20 min trichloroacetic acid washes and rinsed with cold absolute ethanol. The radioactivity bound to casein was counted in a Packard liquid scintillation counter. The protein content was estimated by the bicinchoninic acid method (Pierce Chemicals).

RNA Isolation and Analysis

RNA was isolated from keratinocyte cultures exposed to various concentrations of CSA or anthralin for 24 h. For *in vivo* studies, RNA was extracted from 6 mm skin

biopsies of pre- and post-treatment psoriatic plaques of 2 patients treated for 1-3 months with 2-7.5 mg/kg/d of CSA. RNA was extracted with acid guanidine thiocyanate-phenol-chloroform (184). RNA concentration was determined by absorption at 260 nm. Equal quantities of RNA were size fractionated by electrophoresis in 1% agarose and then transferred to nitrocellulose or Genescreen (New England Nuclear) by following standard protocols (185). Hybridization probes were made by either nick translation (185) or random priming with ^{32}P dCTP using the random primed DNA labeling kit obtained from Boehringer Mannheim. A full length cDNA clone for TGF-α (186) was obtained from Dr. Rik Derynck. A 40 base synthetic oligonucleotide of human β-actin (Oncogene Science, Manhasset, NY) was 5'-end labeled with γ-^{32}P-ATP using T4 polynucleotide kinase (New England Biolaboratories, Beverly, MA). A c-myc specific cDNA probe was a generous gift from Dr. Wilson Miller (Sloan Kettering Memorial Hospital, New York, NY (187)). Hybridization was carried out at 45°C under high stringency conditions using 50% formamide. The membranes were washed and developed on XAR-5 film at -70°C using Cronex intensifying screens. Blots were scanned by optical densitometry.

Measurement of TGF-α Protein

The amount of TGF-α secreted into 4 ml of KGM by confluent cultured keratinocytes grown in the presence and absence of CSA (5 μg/ml), was assayed using a TGF-α radioimmunoassay (91). A standard curve was obtained using synthetic TGF-α at 5 different concentrations.

Receptor Binding Assays

Assays were performed as described under Chapter 2 Receptor Binding Assays. Keratinocytes were incubated with KGM containing DMSO carrier, 5 μg/ml CSA, or 0.01 to 10 μg/ml anthralin for 24 h. Plates were washed 2 x 90 min at 37°C before binding as described above. For Scatchard analysis, anthralin was used at a concentration of 1.0

μg/ml.

Immunohistochemistry

Pre- and post-treatment biopsies of psoriatic plaques were taken from 10 patients treated for 1-3 months with 2-7.5 mg/kg/d CSA. Immunohistochemical staining was performed as described above using mouse monoclonal antibody A1.5 directed against TGF-α (26).

References

1. Krueger, G.G., P.R. Bergstresser, N.J. Lowe, J.J. Voorhees, and G.D. Weinstein. 1984. Psoriasis. *J. Am. Acad. Dermatol.* 11:937.

2. Leczinsky, C.G. 1948. The incidence of arthropathy in a 10-year series of psoriasis cases. *Acta. Dermato-venereologica* 28:483.

3. Fry, L. 1988. Psoriasis. *Br. J. Dermatol.* 119:445.

4. Wuepper, K.D., S.N. Coulter, and A. Haberman. 1990. Psoriasis vulgaris: A genetic approach. *J. Invest. Dermatol.* 95:2S.

5. Burks, J.W., and H. Montgomery. 1943. Histopathologic study of psoriasis. *Arch Dermatol.* 48:479.

6. Van Scott, E.J., and T.M. Ekel. 1963. Kinetics of hyperplasia in psoriasis. *Arch Dermatol.* 88:373.

7. Weinstein, G.D., J.L. McCullough, and P.A. Ross. 1985. Cell kinetic basis for pathophysiology of psoriasis. *J. Invest. Dermatol.* 85:579.

8. Mansbridge, J.N., and A.M. Knapp. 1987. Changes in keratinocyte maturation during wound healing. *J. Invest. Dermatol.* 89:253.

9. Gottlieb, A.B., B. Lifshitz, S.M. Fu, L. Staiano-Coico, C.Y. Wang, and D.M. Carter.

1986. Expression of HLA-DR molecules by keratinocytes and presence of Langerhans cells in the dermal infiltrate of active psoriatic plaques. *J. Exp. Med.* 164:1013.

10. Gottlieb, A.B. 1988. Immunologic mechanisms in psoriasis. *J. Am. Acad. Dermatol.* 18:1376.

11. Baker, B.S., A.F. Swain, L. Fry, and H. Valdimarsson. 1984. Epidermal T lymphocytes and HLA-DR expression in psoriasis. *Br. J. Dermatol.* 11:555.

12. Kaplan, G., M.D. Witmer, I. Nath, R.M. Steinman, S. Laal, H.K. Prasad, E.N. Sarno, U. Elvers, and Z.A. Cohn. 1986. Influence of delayed immune reactions on human epidermal keratinocytes. *Proc. Natl. Acad. Sci. USA* 83:3469.

13. Nathan, C.F., G. Kaplan, W.R. Levis, A. Nusrat, M.D. Witmer, S.A. Sherwin, C.K. Job, C.R. Horowitz, R.M. Steinman, and Z.A. Cohn. 1986. Local and systemic effects of intradermal recombinant interferon-gamma in patients with lepromatous leprosy. *N. Engl. J. Med.* 315:6.

14. Rheinwald, J.G., and H. Green. 1975. Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. *Cell* 6:331.

15. Peehl, D.M., and R.G. Ham. 1980. Growth and differentiation of human keratinocytes with small amounts of dialyzed serum. *In Vitro Cell. Dev. Biol.* 16:526.

16. Boyce, S.T., and R.G. Ham. 1983. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J. Invest. Dermatol.* 81:33s.

17. Gottlieb, A.B., A.D. Luster, D.N. Posnett, and D.M. Carter. 1988. Detection of a gamma interferon-induced protein IP-10 in psoriatic plaques. *J. Exp. Med.* 168:941.

18. Griffiths, C.E.M., J.J. Voorhees, and B.J. Nickoloff. 1989. Characterization of intercellular adhesion molecule-1 and HLA-DR expression in normal and inflamed skin: Modulation by recombinant gamma interferon and tumor necrosis factor. *J. Am. Acad. Dermatol.* 20:617.

19. Dustin, M.I., R. Rothlein, A.K. Bhan, C.A. Dinarello, and T.A. Springer. 1986. Induction by IL-1 and interferon, tissue distribution, biochemistry and function of a natural adherence molecule (ICAM-1). *J. Immunol.* 137:245.

20. Krueger, J.G., J.F. Krane, D.M. Carter, and A.B. Gottlieb. 1990. Role of growth factors, cytokines, and their receptors in the pathogenesis of psoriasis. *J. Invest. Dermatol.* 94:135S.

21. Nickoloff, B.J., T.Y. Basham, T.C. Merrigan, and V.B. Morhenn. 1984. Antiproliferative effects of recombinant α- and γ-interferons on cultured human keratinocytes. *Lab. Invest.* 51:697.

22. Fierlbeck, G., G. Rasner, and G. Muler. 1990. Psoriasis induced at injection site by recombinant gamma interferon. *Arch Dermatol.* 126:351.

23. Ansel, J., P. Perry, J. Brown, D. Damm, T. Phan, C. Hart, T. Luger, and S. Hefeneider. 1990. Cytokine modulation of keratinocyte cytokines. *J. Invest. Dermatol.* 94:101S.

24. Hancock, G.E., G. Kaplan, and Z.A. Cohn. 1988. Keratinocyte growth regulation by the products of immune cells. *J. Exp. Med.* 168:1395.

25. Grossman, R.M., J.G. Krueger, D. Yourish, A. Granelli-Piperno, D.P. Murphy, L.T. May, T.S. Kupper, P.B. Sehgal, and A.B. Gottlieb. 1989. Interleukin 6 is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. *Proc. Natl. Acad. Sci. USA* 86:6367.

26. Gottlieb, A.B., C.K. Chang, D.N. Posnett, B. Fanelli, and J.P. Tam. 1988. Detection of transforming growth factor alpha in normal, malignant, and hyperproliferative human keratinocytes. *J. Exp. Med.* 167:670.

27. Elder, J.T., G.J. Fisher, P.B. Lindquist, G.L. Bennett, M.R. Pittelkow, R.J. Coffey, L. Ellingsworth, R. Derynck, and J.J. Voorhees. 1989. Overexpression of transforming growth factor alpha in psoriatic epidermis. *Science* 243:811.

28. Cooper, K.D., C. Hammerberg, O. Baadsgaard, J.T. Elder, L.S. Chan, D.N. Sauder, J.J. Voorhees, and G.J. Fisher. 1990. IL-1 activity is reduced in psoriatic skin: Decreased IL-1α and increased non-functional IL-1β. *J. Immunol.* 144:4593.

29. Pardee, A.B. 1989. G_1 events and regulation of cell proliferation. *Science* 246:603.

30. Krane, J.F., D.P. Murphy, A.B. Gottlieb, D.M. Carter, C.E. Hart, and J.G. Krueger. 1991. Increased dermal expression of platelet-derived growth factor receptors in growth-activated skin wounds and psoriasis. *J. Invest. Dermatol.* 96:983.

31. Maciag, T., R.E. Nemore, R. Weinstein, and B.A. Gilchrest. 1981. An endocrine approach to the control of epidermal growth: Serum-free cultivation of human keratinocytes. *Science* 211:1452.

32. Tsao, M.C., B.J. Walthall, and R.G. Ham. 1982. Clonal growth of normal human epidermal keratinocytes in a defined medium. *J. Cell. Physiol.* 110:219.

33. Shipley, G.D., W.W. Keeble, J.E. Hendrickson, R.J. Coffey, and M.R. Pittelkow. 1989. Growth of normal human keratinocytes and fibroblasts in serum-free medium is stimulated by acidic and basic fibroblast growth factor. *J. Cell Physiol.* 138:511.

34. O'Keefe, E.J., M.L. Chiu, and R.E. Payne. 1988. Stimulation of growth of keratinocytes by basic fibroblast growth factor. *J. Invest. Dermatol.* 90:767.

35. Wille, J.J., M.R. Pittelkow, G.D. Shipley, and R.E. Scott. 1984. Integrated control of growth and differentiation of normal human prokeratinocytes cultured in serum-free medium: Clonal analyses, growth kinetics, and cell cycle studies. *J. Cell. Physiol.* 121:31.

36. Cohen, S. 1962. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J. Biol. Chem.* 237:1555.

37. Cohen, S., and G.A. Elliott. 1963. The stimulation of epidermal keratinization by a protein isolated from the submaxillary gland of the mouse. *J. Invest. Dermatol.* 40:1.

38. Rheinwald, J.G., and H. Green. 1977. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature* 265:421.

39. Barrandon, Y., and H. Green. 1987. Cell migration is essential for sustained growth of keratinocyte colonies: The roles of transforming growth factor-alpha and epidermal growth factor. *Cell* 50:1131.

40. Barrandon, Y., and H. Green. 1985. Cell size as a determinant of the clone-forming ability of human keratinocytes. *Proc. Natl. Acad. Sci. USA* 82:5390.

41. Ponec, M., A. Weerheim, J. Kempenaar, and J. Boonstra. 1988. Proliferation and differentiation of human squamous carcinoma cell lines and normal keratinocytes: Effects of epidermal growth factor, retinoids, and hydrocortisone. *In Vitro* 24:764.

42. Coffey, R.J., R. Derynck, J.N. Wilcox, T.S. Bringman, A.S. Goustin, H.L. Moses, and M.R. Pittelkow. 1987. Production and auto-induction of transforming growth factor-alpha in human keratinocytes. *Nature* 328:817.

43. Pittelkow, M.R., P.B. Lindquist, R.T. Abraham, R. Graves-Deal, R. Derynck, and R.J. Coffey. 1989. Induction of transforming growth factor-alpha expression in human keratinocytes by phorbol esters. *J. Biol. Chem.* 264:5164.

44. Krueger, J.G., J.F. Krane, A.B. Gottlieb, D. Yourish, P.B. Sehgal, L.T. May, S. Decker, and T.S. Kupper. 1989. Cytokine modulation of epithelial function: Contrasting effects of interleukin 6/β_2-interferon and γ-interferon on human keratinocyte growth and differentiation (abstr). *J. Invest. Dermatol.* 92:464.

45. Bjorge, J.D., A.J. Paterson, and J.E. Kudlow. 1989. Phorbol ester or epidermal growth factor (EGF) stimulates the concurrent accumulation of mRNA for the EGF receptor and its ligand transforming growth factor-α in a breast cancer cell line. *J. Biol.*

Chem. 264:4021.

46. Kupper, T.S. 1990. The activated keratinocyte: A model for inducible cytokine production by non-bone marrow-derived cells in cutaneous inflammatory and immune responses. *J. Invest. Dermatol.* 94:146S.

47. Di Fiore, P.P., J.H. Pierce, T.P. Fleming, R. Hazan, A. Ullrich, C.R. King, J. Schlessinger, and S.A. Aaronson. 1987. Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells. *Cell* 51:1063.

48. Nanney, L.B., C.M. Stoscheck, M. Magid, and L.E. King. 1986. Altered [^{125}I]epidermal growth factor binding and receptor distribution in psoriasis. *J. Invest. Dermatol.* 86:260.

49. Vassar, R., and E. Fuchs. 1991. Transgenic mice provide new insights into the role of TGF-α during epidermal development and differentiation. *Genes and Development* 5:714.

50. King, L.E., R.E. Gates, C.M. Stoscheck, and L.B. Nanney. 1990. The EGF/TGFα receptor in skin. *J. Invest. Dermatol.* 94:164S.

51. Gentleman, S., T.M. Martensen, J.J. Digiovanna, and G.J. Chader. 1984. Protein tyrosine kinase and protein phosphotyrosine phosphatase in normal and psoriatic skin. *Biochem. Biophys. Acta* 798:53.

52. Bartel, R.L., C.L. Marcelo, and J.J. Voorhees. 1987. Partial characterization of phospholipase C activity in normal, psoriatic uninvolved, and lesional epidermis. *J. Invest. Dermatol.* 88:447.

53. Horn, F., F. Marks, G.J. Fisher, C.L. Marcelo, and J.J. Voorhees. 1987. Decreased protein kinase C activity in psoriatic versus normal epidermis. *J. Invest. Dermatol.* 88:220.

54. Fisher, G.J., H.S. Talwar, A. Tavakkol, J. Esmann, J.J. Baldassare, J.T. Elder, C.E.M. Griffiths, O. Baadsgaard, K.D. Cooper, and J.J. Voorhees. 1990. Phosphoinositide-mediated signal transduction in normal and psoriatic epidermis. *J. Invest. Dermatol.* 95:15S.

55. Pledger, W.J., C.D. Stiles, H.N. Antoniades, and C.D. Scher. 1978. An ordered sequence of events is required before BALB/c-3T3 cells become committed to DNA synthesis. *Proc. Natl. Acad. Sci. USA* 75:2839.

56. Shipley, G.D., and M.R. Pittelkow. 1987. Control of growth and differentiation in vitro of human keratinocytes cultured in serum-free medium. *Arch Dermatol.* 123:1541a.

57. Krane, J.F., D.P. Murphy, D.M. Carter, and J.G. Krueger. 1991. 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. *J. Invest. Dermatol.* 96:419.

58. Krane, J.F., A.B. Gottlieb, D.M. Carter, and J.G. Krueger. 1992. The insulin-like growth factor I receptor is overexpressed in psoriatic epidermis, but is differentially regulated from the epidermal growth factor receptor. *J. Exp. Med.* 175:1081.

59. Daughaday, W.H., and P. Rotwein. 1989. Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations.

Endocr. Rev. 10:68.

60. Massague, J., and M.P. Czech. 1982. The subunit structures of two distinct receptors for insulin-like growth factors I and II and their relationship to the insulin receptor. *J. Biol. Chem.* 257:5038.

61. Czech, M.P. 1989. Signal transmission by the insulin-like growth factors. *Cell* 59:235.

62. Ullrich, A., A. Gray, A.W. Tam, T. Yang-Feng, M. Tsubokawa, C. Collins, W. Henzel, T. Le Bon, S. Kathuria, E. Chen, S. Jacobs, U. Francke, J. Ramachandran, and Y. Fujita-Yamaguchi. 1986. Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J.* 5:2503.

63. Lammers, R., A. Gray, J. Schlessinger, and A. Ullrich. 1989. Differential signalling potential of insulin- and IGF-1-receptor cytoplasmic domains. *EMBO J.* 8:1369.

64. Werner, H., M. Woloschak, M. Adamo, Z. Shen-Orr, C.T. Roberts, and D. LeRoith. 1989. Developmental regulation of the rat insulin-like growth factor I receptor gene. *Proc. Natl. Acad. Sci. USA* 86:7451.

65. Aaronson, S.A., J.S. Rubin, P.W. Finch, J. Wong, C. Marchese, J. Falco, W.G. Taylor, and M.H. Kraus. 1990. Growth factor-regulated pathways in epithelial cell proliferation. *Am. Rev. Respir. Dis.* 142:S7.

66. Hansson, H.-A., R. Jonsson, and K. Petruson. 1988. Transiently increased insulin-like growth factor I immunoreactivity in UVB-irradiated mouse skin. *J. Invest. Dermatol.*

91:328.

67. Misra, P., B.J. Nickoloff, V.B. Morhenn, R.L. Hintz, and R.G. Rosenfeld. 1986. Characterization of insulin-like growth factor-I/somatomedin-C receptors on human keratinocyte monolayers. *J. Invest. Dermatol.* 87:264.

68. Nickoloff, B.J., P. Misra, V.B. Morhenn, R.L. Hintz, and R.G. Rosenfeld. 1988. Further characterization of the keratinocyte somatomedin-C/insulin-like growth factor I (SM-C/IGF-1) receptor and the biological responsiveness of cultured keratinocytes to SM-C/IGF-1. *Dermatologica* 177:265.

69. Pfeifle, B., and H. Ditschuneit. 1983. Two separate receptors for insulin and insulin-like growth factors on arterial smooth muscle cells. *Exp. Clin. Endicrinol.* 81:280.

70. Rosenfeld, R.G., and L.A. Dollar. 1982. Characterization of the somatomedin-C/insulin-like growth factor I (SM-C/IGF I) receptor on cultured human fibroblast monolayers: regulation of receptor concentrations by SM-C/IGF I and insulin. *J. Clin. Endicrinol. Metab.* 55:434.

71. Tong, P.S., N.N. Horowitz, and L.A. Wheeler. 1990. Trans retinoic acid enhances the growth response of epidermal keratinocytes to epidermal growth factor and transforming growth factor beta. *J. Invest. Dermatol.* 94:126.

72. Green, M.R., and J.R. Couchman. 1985. Differences in human skin between the epidermal growth factor receptor distribution detected by EGF binding and monoclonal antibody recognition. *J. Invest. Dermatol.* 85:239.

73. Kull, F.C. ., S. Jacobs, Y.-F. Su, M.E. Svoboda, J.J. Van Wyk, and P. Cuatrecasas. 1983. Monoclonal antibodies to receptors for insulin and somatomedin-C. *J. Biol. Chem.* 258:6561.

74. Jacobs, S., S. Cook, M.E. Svoboda, and J.J. Van Wyk. 1986. Interaction of the monoclonal antibodies alpha-IR-1 and alpha-IR-3 with insulin and somatomedin-C receptors. *Endocrinology* 118:223.

75. Catanese, V.M., F. Grigorescu, G.L. King, and R.C. Kahn. 1986. The human erythrocyte insulin-like growth factor I receptor: charecterization and demonstration of ligand-stimulated autophosphorylation. *J. Clin. Endicrinol. Metab.* 62:692.

76. Furlanetto, R.W., J.N. DiCarlo, and C. Wisehart. 1987. The type II insulin-like growth factor receptor does not mediate deoxyribonucleic acid synthesis in human fibroblasts. *J. Clin. Endicrinol. Metab.* 64:1142.

77. Shoyab, M., J.E. DeLarco, and G.J. Todaro. 1979. Biologically active phorbol esters specifically alter affinity of epidermal growth factor membrane receptors. *Nature* 279:387.

78. Fearn, J.C., and A.C. King. 1985. EGF receptor affinity is regulated by intracellular calcium and protein kinase C. *Cell* 40:991.

79. Davis, R.J., and M.P. Czech. 1984. Tumor-promoting phorbol diesters mediate phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* 259:8545.

80. Cochet, C., G.N. Gill, J. Meisenhelder, J.A. Cooper, and T. Hunter. 1984. C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth

factor-stimulated tyrosine protein kinase activity. *J. Biol. Chem.* 259:2553.

81. Liu, C., and T.E. Hermann. 1978. Characterization of ionomycin as a calcium ionophore. *J. Biol. Chem.* 253:5982.

82. Bird, T.A., and J. Saklatvala. 1989. IL-1 and TNF transmodulate epidermal growth factor receptors by a protein kinase C-independent mechanism. *J. Immunol.* 142:126.

83. Pittelkow, M.R. 1985. Somatomedin C is a growth factor for human prokeratinocytes in vitro (abstr). *Br. J. Dermatol.* 113:769.

84. Ristow, H.-J., and T.O. Messmer. 1988. Basic fibroblast growth factor and insulin-like growth factor I are strong mitogens for cultured mouse keratinocytes. *J. Cell. Physiol.* 137:277.

85. O'Keefe, E.J., and M.L. Chiu. 1988. 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.

86. O'Keefe, E.J., T. Battin, and R. Payne. 1982. Epidermal growth factor receptor in human epidermal cells: Direct demonstration in cultured cells. *J. Invest. Dermatol.* 78:482.

87. Werner, H., B. Stannard, M.A. Bach, D. LeRoith, and C.T. Roberts. 1990. Cloning and characterization of the proximal promoter region of the rat insulin-like growth factor I (IGF-I) receptor gene. *Biochem. Biophys. Res. Commun.* 169:1021.

88. Zendegui, J.G., W.H. Inman, and G. Carpenter. 1988. Modulation of the mitogenic

response of an epidermal growth factor-dependent keratinocyte cell line by dexamethasone, insulin, and transforming growth factor-ß. *J. Cell. Physiol.* 136:257.

89. DeLapp, N.W., and D.K. Dieckman. 1990. 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. *J. Invest. Dermatol.* 94:777.

90. Nickoloff, B.J., R.S. Mitra, B.L. Riser, V.M. Dixit, and J. Varani. 1988. Modulation of keratinocyte motility: Correlation with production of extracellular matrix molecules in response to growth promoting and antiproliferative factors. *Am. J. Pathol.* 132:543.

91. Nickoloff, B.J., and R.S. Mitra. 1989. Inhibition of ^{125}I-epidermal growth factor binding to cultured keratinocytes by antiproliferative molecules gamma interferon, cyclosporin A, and transforming growth factor-beta. *J. Invest. Dermatol.* 93:799.

92. Stewart, A.J., M.D. Johnson, F.E.B. May, and B.R. Westley. 1990. Role of insulin-like growth factors and the type I insulin-like growth factor receptor in the estrogen-stimulated proliferation of human breast cancer cells. *J. Biol. Chem.* 265:21172.

93. Braverman, I.M., and A. Yen. 1977. Ultrastructure of the human dermal microcirculation: II. The capillary loops of the dermal papillae. *J. Invest. Dermatol.* 68:44.

94. Camisa, C. 1989. Somatostatin and a long-acting analogue, octreotide acetate. Relevance to dermatology. *Arch. Dermatol.* 125:407.

95. Burgess, W.H., and T. Maciag. 1989. The heparin-binding (fibroblast) growth factor family of proteins. *Ann. Rev. Biochem.* 58:575.

96. Gospodarowicz, D. 1975. Purification of a fibroblast growth factor from bovine pituitary. *J. Biol. Chem.* 250:2515.

97. Bovi, P.D., A.M. Curatola, F.G. Kern, A. Greco, M. Ittmann, and C. Basilico. 1987. An oncogene isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. *Cell* 50:729.

98. Zhan, X., B. Bates, X. Hu, and M. Goldfarb. 1988. The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. *Mol. Cell. Biol.* 8:3487.

99. Marics, I., J. Adelaide, F. Raybaud, M. Mattei, F. Coulier, J. Planche, O. Lapeyriere, and D. Birnbaum. 1989. Characterization of the HST-related FGF 6 gene, a new member of the fibroblast growth factor gene family. *Oncogene* 4:335.

100. Rubin, J.S., H. Osada, P.W. Finch, W.G. Taylor, S. Rudikoff, and S.A. Aaronson. 1989. Purification and characterization of a newly identified growth factor specific for epithelial cells. *Proc. Natl. Acad. Sci. USA* 86:802.

101. Finch, P.W., J.S. Rubin, T. Miki, D. Ron, and S.A. Aaronson. 1989. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science* 245:752.

102. Marchese, C., J.S. Rubin, D. Ron, A. Faggioni, M.R. Torrisi, A. Messina, L. Frati, and S.A. Aaronson. 1990. Human keratinocyte growth factor activity on proliferation and differentiation of human keratinocytes: Differentiation response distinguishes KGF from EGF family. *J. Cell Physiol.* 144:326.

103. Gospodarowicz, D., J. Plouet, and B. Malerstein. 1990. Comparison of the ability of basic and acidic fibroblast growth factor to stimulate the proliferation of an established keratinocyte cell line: Modulation of their biological effects by heparin, transforming growth factor β (TGFβ), and epidermal growth factor (EGF). *J. Cell Physiol.* 142:325.

104. Falco, J.P., W.G. Taylor, P.P. Di Fiore, B.E. Weissman, and S.A. Aaronson. 1988. Interactions of growth factors and retroviral oncogenes with mitogenic signal transduction pathways of Balb/MK keratinocytes. *Oncogene* 2:573.

105. Halaban, R., R. Langdon, N. Birchall, C. Cuono, A. Baird, G. Scott, G. Moellmann, and J. McGuire. 1988. Basic fibroblast growth factor from human keratinocytes is a natural mitogen for melanocytes. *J. Cell Biol.* 107:1611.

106. Abraham, J.A., A. Mergia, J.L. Whang, A. Tumolo, J. Friedman, K.A. Hjorild, D. Gospodarowicz, and J.C. Fiddes. 1986. Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science* 233:545.

107. Flaumenhaft, R., D. Moscatelli, O. Saksela, and D.B. Rifkin. 1989. Role of extracellular matrix in the action of basic fibroblast growth factor: Matrix as a source of growth factor for long-term stimulation of plasminogen activator production and DNA synthesis. *J. Cell Physiol.* 140:75.

108. Lee, P.L., D.E. Johnson, L.S. Cousens, V.A. Fried, and L.T. Williams. 1989. Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. *Science* 245:57.

109. Hou, J., M. Kan, K. McKeehan, G. McBride, P. Adams, and W.L. McKeehan. 1991. Fibroblast growth factor receptors from liver vary in three structural domains. *Science* 251:665.

110. Dionne, C.A., G. Crumley, F. Bellot, J.M. Kaplow, G. Searfoss, M. Ruta, W.H. Burgess, M. Jaye, and J. Schlessinger. 1990. Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. *EMBO J.* 9:2685.

111. Ruta, M., W.H. Burgess, D. Givol, J. Epstein, N. Neiger, J. Kaplow, G. Crumley, C.A. Dionne, M. Jaye, and J. Schlessinger. 1989. Receptor for acidic fibroblast growth factor is related to the tyrosine kinase encoded by the fms-like gene (FLG). *Proc. Natl. Acad. Sci. USA* 86:8722.

112. Avivi, A., Y. Zimmer, A. Yayon, Y. Yarden, and D. Givol. 1991. Flg-2, a new member of the family of fibroblast growth factor receptors. *Oncogene* 6:1089.

113. Moscatelli, D. 1987. High and low affinity binding sites for basic fibroblast growth factor on cultured cells: Absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J. Cell Physiol.* 131:123.

114. Bottaro, D.P., J.S. Rubin, D. Ron, P.W. Finch, C. Florio, and S.A. Aaronson. 1990. Characterization of the receptor for keratinocyte growth factor. *J. Biol. Chem.* 265:12767.

115. Ross, R. 1989. Platelet-derived growth factor. *Lancet* 1:1179.

116. Clemmons, D.R. 1984. Multiple hormones stimulate the production of somatomedin by cultured human fibroblasts. *J. Clin. Endocrinol. Metab.* 58:850.

117. Bronzert, D.A., P. Pantazis, H.N. Antoniades, A. Kasid, N. Davidson, and R.B. Dickson. 1987. Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. *Proc. Natl. Acad. Sci. USA* 84:5763.

118. Damm, D., G. Shipley, C. Hart, and J. Ansel. 1990. The differential regulation of PDGF-A and PDGF-B in normal human keratinocytes (abstr). *J. Invest. Dermatol.* 94:517.

119. Hart, C.E., and D.F. Bowen-Pope. 1990. Platelet-derived growth factor receptor: Current views of the two-subunit model. *J. Invest. Dermatol.* 94:53S.

120. Gu, X.-F., F. Raynaud, and D. Evain-Brion. 1988. Increased chemotactic and mitogenic response of psoriatic fibroblasts to platelet-derived growth factor. *J. Invest. Dermatol.* 91:599.

121.Bowen-Pope, D.F., and R. Ross. 1982. Platelet-derived growth factor. II. Specific binding to cultured cells. *J. Biol. Chem.* 257:5161.

122. Murphy, D.P., B. Smoller, A.B. Gottlieb, A. Hsu, D.M. Carter, and J.G. Krueger. 1990. Chronic, non-healing skin ulcers display a growth-activated epidermis (regenerative maturation) with high level TGF-alpha and EGF-receptor expression (abstr). *J. Invest. Dermatol.* 94:557.

123. Greenhalgh, D.G., K.H. Sprugel, M.J. Murray, and R. Ross. 1990. PDGF and FGF stimulate wound healing in the genetically diabetic mouse. *Am. J. Pathol.* 136:1235.

124. Hart, C.E., R.A. Seifert, R. Ross, and D.F. Bowen-Pope. 1987. Synthesis, phosphorylation, and degradation of multiple forms of the platelet-derived growth factor receptor studied using a monoclonal antibody. *J. Biol. Chem.* 262:10780.

125. Smits, A., M. Hermansson, M. Nister, I. Karnushina, C.-H. Heldin, B. Westermark, and K. Funa. 1989. Rat brain capillary endothelial cells express functional PDGF B-type receptors. *Growth Factors* 2:1.

126. Sehgal, P.B. 1990. Interleukin-6: Molecular pathophysiology. *J. Invest. Dermatol.* 94:2S.

127. Krane, J.F., D.P. Murphy, D.M. Carter, and J.G. Krueger. 1991. 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. *J. Invest. Dermatol.* 96:419.

128. Saiag, P., B. Coulomb, C. Lebreton, E. Bell, and L. Dubertret. 1985. Psoriatic fibroblasts induce hyperproliferation of normal keratinocytes in a skin equivalent model in vitro. *Science* 230:669.

129. Menter, A., and J.N.W.N. Barker. 1991. Psoriasis in practice. *Lancet* 338:231.

130. Goeckerman, W.H. 1925. Treatment of psoriasis. *Northwest Med.* 24:229.

131. Khandke, L., J.F. Krane, R. Ashinoff, L. Staiano-Coico, A. Granelli-Piperno, A.D. Luster, D.M. Carter, J.G. Krueger, and A.B. Gottlieb. 1991. Cyclosporine in psoriasis treatment: Inhibition of keratinocyte cell-cycle progression in G1 independent of effects on transforming growth factor-α/epidermal growth factor receptor pathways. *Arch Dermatol.* 127:1172.

132. Gottlieb, A.B., L. Khandke, J.F. Krane, L. Staiano-Coico, R. Ashinoff, and J.G. Krueger. 1992. Anthralin decreases keratinocyte TGF-α expression and EGF receptor binding in vitro. *J. Invest. Dermatol.* 98:680.

133. Gottlieb, A.B., R.M. Grossman, L. Khandke, D.M. Carter, P.B. Sehgal, S.M. Fu, A. Granelli-Piperno, M. Rivas, L. Barazani, and J.G. Krueger. 1992. Studies of the effect of cyclosporine in psoriasis in vivo: Combined effects on activated T lymphocytes and epidermal regenerative maturation. *J. Invest. Dermatol.* 98:302.

134. Kemeny, L., T. Ruzicka, and O. Braun-Falco. 1990. Dithranol: A review of the mechanism of action in the treatment of psoriasis vulgaris. *Skin Pharmacol.* 3:1.

135. Ellis, C.N., D.C. Gorsulowsky, T.A. Hamilton, J.K. Billings, M.D. Brown, J.T. Headington, K.D. Cooper, O. Baadsgaard, E.A. Duell, T.M. Annesley, J.G. Thurcotte, and J.J. Voorhees. 1986. Cyclosporine improves psoriasis in a double-blind study. *JAMA* 256:3110.

136. Griffiths, C.E.M., A.V. Powles, and J.N. Leonard. 1986. Clearance of psoriasis with low dose cyclosporin. *BMJ* 293:731.

137. Ellis, C.N., M.S. Fradlin, J.M. Messana, M.D. Brown, M.T. Siegel, A.H. Hartley,

L.L. Rocher, S. Wheeler, T.A. Hamilton, T.G. Parish, M. Ellis-Madu, E. Duell, T.M. Annesley, and J.J. Voorhees. 1991. Cyclosporin for plaque-type psoriasis. Results of a multidose, double-blind trial. *N. Engl. J. Med.* 324:277.

138. Meuller, W., and B. Herrmann. 1979. Cyclosporin A for psoriasis. *N. Engl. J. Med.* 301:555.

139. Joost, T.V., J.D. Bos, F. Heule, and M.M.H.M. Meinardi. 1988. Low-dose cyclosporin A in severe psoriasis: A double-blind study. *Br. J. Dermatol.* 118:183.

140. Bos, J.D. 1988. The pathomechanisms of psoriasis: The skin immune system and cyclosporin. *Br. J. Dermatol.* 118:141.

141. Urabe, A., J. Kanitakis, J. Viac, and J. Thivolet. 1989. Cyclosporin A inhibits directly in vivo keratinocyte proliferation of living human skin. *J. Invest. Dermatol.* 92:755.

142. Furue, M., A.A. Gaspari, and S.I. Katz. 1988. The effect of cyclosporin A on epidermal cells, II: Cyclosporin A inhibits proliferation of normal and transformed keratinocytes. *J. Invest. Dermatol.* 90:796.

143. Fisher, G.J., E.A. Duell, B.J. Nickoloff, T.M. Annesley, J.K. Kowalke, C.N. Ellis, and J.J. Voorhees. 1988. Levels of cyclosporine in epidermis of treated psoriasis patients differentially inhibit growth of keratinocytes cultured in serum free versus serum containing media. *J. Invest. Dermatol.* 91:142.

144. Cooper, K.D., O. Baadsgaard, C.N. Ellis, E.A. Duell, and J.J. Voorhees. 1990. Mechanisms of cyclosporin A inhibition of antigen presenting activity in uninvolved and

lesional psoriatic epidermis. *J. Invest. Dermatol.* 94:649.

145. Yoshimura, N., T. Oka, S.C. Clark, and B.D. Kahan. 1987. The inhibition of interleukin 2 (IL-2) gene expression at the level of messenger RNA by in vivo cyclosporine treatment in kidney transplant recipients. *Transplant Proc.* 19:3510.

146. Granelli-Piperno, A., K. Inaba, and R.M. Steinman. 1984. Stimulation of lymphokine release from T lymphoblasts: Requirement for mRNA synthesis and inhibition by cyclosporin A. *J. Exp. Med.* 160:1792.

147. Granelli-Piperno, A., L. Andrus, and R.M. Steinman. 1986. Lymphokine and nonlymphokine mRNA levels in stimulated human T cells: Kinetics, mitogen requirements, and effects of cyclosporin A. *J. Exp. Med.* 163:922.

148. Granelli-Piperno, A., M. Keane, and R.M. Steinman. 1988. Evidence that cyclosporin A inhibits cell-mediated immunity primarily at the level of the T lymphocyte rather than the accessory cells. *Transplantation* 46:53S.

149. Granelli-Piperno, A. 1988. In situ hybridization for interleukin 2 and interleukin 2 receptor mRNA in T cells activated in the presence or absence of cyclosporin A. *J. Exp. Med.* 168:1649.

150. Cooper, K.D., J.J. Voorhees, G.J. Fisher, L.S. Chan, and A.K. Gupta. 1990. Effects of cyclosporine on immunologic mechanisms in psoriasis. *J. Am. Acad. Dermatol.* 23:1318.

151. Furue, M., and S.I. Katz. 1988. The effects of cyclosporin A on epidermal cells, I:

Cyclosporin inhibits accessory cell functions of epidermal Langerhans cells in vitro. *J. Immunol.* 140:4139.

152. Nickoloff, B.J., G.J. Fisher, R.S. Mitra, and J.J. Voorhees. 1988. Additive and synergistic antiproliferative effects of cyclosporin A and gamma interferon on cultured human keratinocytes. *Am. J. Pathol.* 131:12.

153. Dykes, P.J., J. Brunt, and R. Marks. 1990. The effect of cyclosporin on human epidermal keratinocytes in vitro. *Br. J. Dermatol.* 122:173.

154. Sharpe, G.R., and C. Fisher. 1990. Time dependent inhibition of human keratinocytes and fibroblasts by cyclosporin A: Effect on keratinocytes at therapeutic blood levels. *Br. J. Dermatol.* 123:207.

155. Squire, B. 1877. Chrysophanic acid as a remedy in skin disease. *BMJ* 1S:199.

156. Liebermann, C., and P. Seidler. 1878. Uber Chrysophansaure im Goapulver. *Ber. Dtsch. Chem. Ges.* 14:1603.

157. Galewsky, E. 1916. Uber Cignolin, ein Ersatzpraparat des Chrysarobins. *Dermatol. Wochenschr.* 62:113.

158. Schroder, J.M., U. Kosfeld, and E. Christophers. 1985. Multifunctional inhibition by anthralin in nonstimulated and chemotactic factor stimulated human neutrophils. *J. Invest. Dermatol.* 85:30.

159. Tucker, W.F.G., S. MacNeil, R.A. Dawson, S. Tomlinson, and S.S. Bleehen. 1986.

An investigation of the ability of antipsoriatic drugs to inhibit calmodulin activity: A possible mode of action of dithranol (Anthralin). *J. Invest. Dermatol.* 87:232.

160. Wollina, U., R. Klinger, R. Wetzker, and B. Knopf. 1989. Calmodulin inhibition by Anthralin? Investigations in vitro and in vivo. *Dermatologica* 179:61.

161. Czernielewski, J., L. Juhlin, S. Shroot, and P. Brun. 1985. Langerhans' cells in patients with psoriasis: Effect of treatment with PUVA, PUVA bath, etretinate and anthralin. *Acta. Dermato-venereologica* 65:97.

162. Chang, A., H. Alkemade, and P.C.M. VandeKerkhof. 1989. Dithranol modulates the leukotriene B4-induced intraepidermal accumulation of polymorphonuclear leukocytes. *J. Invest. Dermatol.* 92:806.

163. Lowe, N.J. 1981. Anthralin. Different concentration effects on epidermal cell DNA synthesis rates in mice and clinical responses in human psoriasis. *Arch Dermatol.* 117:698.

164. Clark, J.M., and P.C. Hanawalt. 1982. Inhibition of DNA replication and repair by anthralin and danthron in cultured human cells. *J. Invest. Dermatol.* 79:18.

165. Holland, D.B., E.J. Wood, W.J. Cunliffe, and D.M. Turner. 1989. Keratin gene expression during the resolution of psoriatic plaques: Effect of dithranol, PUVA, etretinate and hydroxyurea regimens. *Br. J. Dermatol.* 120:9.

166. Fuchs, J., W.H. Nitschmann, and L. Packer. 1990. The antipsoriatic compound anthralin influences bioenergetic parameters and redox properties of energy transducing membranes. *J. Invest. Dermatol.* 94:71.

167. Lotti, T., P. Bonan, G. Cannarozzo, A.M. Fedi, and E. Panconesi. 1990. Antipsoriatic therapies inhibit epidermal plasminogen activator activity. *Intl. J. Dermatol.* 29:528.

168. Staiano-Coico, L., R.T. Higgins, Z. Darzynkiewicz, M. Kimmel, A.B. Gottlieb, I. Pagan-Charry, M.R. Madden, J.L. Finkelstein, and J.M. Hefton. 1986. Human keratinocyte culture: Identification and staging of epidermal cell subpopulations. *J. Clin. Invest.* 77:396.

169. Yuspa, S.H., T. Ben, H. Hennings, and U. Lichti. 1980. Phorbol ester tumor promoters induce epidermal transglutaminase activity. *Biochem. Biophys. Res. Commun.* 97:700.

170. Newburger, A.E., G.D. Weinstein, and J.L. McCullough. 1978. Biological and biochemical actions of methotrexate in psoriasis. *J. Invest. Dermatol.* 70:183.

171. Nickoloff, B.J. 1991. The cytokine network in psoriasis. *Arch Dermatol.* 127:871.

172. Gottlieb, A.B. 1990. Immunologic mechanisms in psoriasis. *J. Invest. Dermatol.* 95:18S.

173. Staiano-Coico, L., A.B. Gottlieb, L. Barazani, and D.M. Carter. 1987. RNA, DNA and cell surface characteristics of lesional and non-lesional psoriatic skin. *J. Invest. Dermatol.* 88:646.

174. Darzynkiewicz, Z., L. Staiano-Coico, and M.R. Melamed. 1981. Increased

mitochondrial uptake of rhodamine 123 during lymphocyte stimulation. *Proc. Natl. Acad. Sci. USA* 78:2383.

175. Katan, M., and P.J. Parker. 1988. Oncogenes and cell control. *Nature* 332:203.

176. Elder, J.T., S.B. Klein, A. Tavakkol, G.J. Fisher, B.J. Nickoloff, and J.J. Voorhees. 1990. Growth factor and proto-oncogene expression in psoriasis. *J. Invest. Dermatol.* 95:7S.

177. Elder, J.T., M.E. Zeigler, S. Wicha, and J.J. Voorhees. 1988. Increased c-myc and c-ras mRNA levels in psoriatic epidermis (abstr). *J. Invest. Dermatol.* 90:555.

178. Gottlieb, A.B., J.G. Krueger, L. Khandke, R.M. Grossman, R.J. Delaney, S.M. Fu, L. Barazani, and D.M. Carter. 1991. Cyclosporine A treatment decreases immune activation in psoriatic plaques without decreasing keratinocyte regenerative maturation (growth activation) (abstr). *J. Invest. Dermatol.* 96:570.

179. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680.

180. Decker, S.J. 1984. Aspects of the metabolism of the epidermal growth factor receptor in A431 human epidermoid carcinoma cells. *Mol. Cell. Biol.* 4:571.

181. Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350.

182. Gottlieb, A.B., R.G. Lahita, N. Chiorazzi, and H.G. Kunkel. 1979. Immune function in systemic lupus erythematosus: Impairment of in vitro T-cell proliferation and in vivo antibody response to exogenous antigen. *J. Clin. Invest.* 63:885.

183. Gottlieb, A.B., L. Mayer, F. Bonetti, D.M. Knowles, J.G. Krueger, H.G. Kunkel, and D.M. Carter. 1985. A membrane protein preferentially expressed by a subpopulation of immature lymphoid cells, epidermal basal keratinocytes and other epithelial stem cells. *J. Am. Acad. Dermatol.* 13:54.

184. Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-pheno-chloroform extraction. *Anal. Biochem.* 162:156.

185. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY. 109-112.

186. Derynck, R. 1986. Transforming growth factor α: Structure and biological activities. *J. Cell. Biochem.* 32:293.

187. Miller, W.H., D. Moy, A. Li, J.F. Grippo, and E. Dmithrovsky. 1990. Retinoic acid induces down-regulation of several growth factors and proto-oncogenes in a human embryonal cancer cell line. *Oncogene* 5:511.

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