Integrin expression during epidermal morphogenesis and wound healing.

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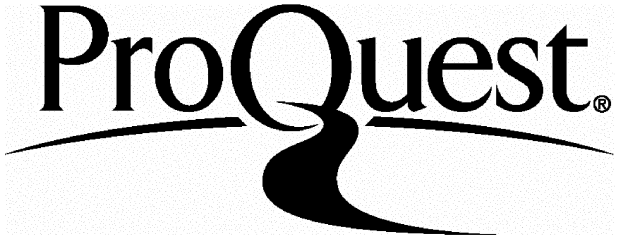

Abstract

The integrins are a family of heterodimeric adhesion receptors expressed by virtually all cells. Integrins can bind cell and extracellular matrix ligands and are involved in signal transduction and migration. The epidermis, the outer protective covering of the body, consists of stratifiied keratinocytes attached to a basement membrane, primarily through integrins. As keratinocytes commit to terminal differentiation, they downregulate integrin function and leave the basement membrane. Our lab has previously shown that integrin dowregulation is an early, required event in commitment to terminal differentiation.

In view of the importance of integrins, and their localization, integrins could be expected to play a role in the establishment and maintenance of the morphological organization of the epidermis. I have examined the pattern of integrin expression during the development of human epidermis and during healing of cutaneous blister wounds.

During development of the epidermis, a single layer of keratinocytes progressively gives rise to multiple suprabasal layers of differentiated keratinocytes. The integrins expressed during development are the same as those in mature skin, but expression is temporally regulated and there are changes in distribution, especially at the onset of stratification. There are no apparent changes in ligand appearance or distribution. I have used skin organ culture to successfully reproduce these events *in vitro.*

During healing of cutaneous suction blisters — in which keratinocytes must migrate to close the wound as well as re-establish the normal architecture — striking, strong suprabasal integrin expression during later stages of wound healing was detected, in contrast to the basally restricted patter seen in unwounded epidermis. Suprabasal expression was also detected in psoriasis, a hyperproliferative disease. I have explored and discounted the possibility that inflammatory cytokines are responsible for this patter. However, keratinocyte-fibroblast organotypic cultures do express integrins suprabasally, and provide a model for further analysis.

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Acknowledgements

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Chapter 1

Introduction

1.1 Human skin

The outermost layer of the human body is the skin. Its primary function is protective, against mechanical damage, desiccation, infection, and environmental agents (e.g. UV radiation). Surrounding the entire body, skin structure varies slightly with body site, but is always composed of three distinct regions, and a basement membrane. A layer of subcutaneous fat forms the innermost layer. The dermis, comprising the majority of the mass of skin, is the next layer and contains blood vessels and nerves. The basement membrane, a specialized extracellular matrix covering the dermis, separates the dermis and epidermis and forms the attachment substrate for the epidermis. The epidermis is composed primarily of keratinocytes, although other accessory cells are also present: melanocytes contribute pigment to the basal layer; Merkel cells convey sensory information through attached nerves; and Langerhans cells have an immune surveillance function.

The ontogeny of skin is discussed in Chapter 3; the healing of skin damage in Chapter 4, and the cytokine/growth factor production and responses of skin in Chapter 5. Here, I will concentrate on the morphology, and the biochemical and structural features, of mature skin. I will also discuss the integrins, and in particular their distribution and function in the epidermis.

1.2 The dermis

The dermis is composed of two anatomically distinct regions, the papillary dermis (named for the epidermal papillary ridges which extend from the epidermis), and an inner layer, the mostly acellular, avascular reticular dermis (reviewed in (Odland 1991)). Although the dermis is primarily composed of collagen I fibers — organized into bundles — collagen III fibers (Meigel *et al*, 1977) and collagen V around blood vessels and at the basement membrane (Konomi *et al*, 1984), are also present. There are also several different glycosaminoglycans, including hyaluronic acid (reviewed in (Comper and Laurent 1978). Several other components are present, including elastin, tenascin (Chiquet-Ehrismann 1991) and fibronectin (Yamada 1989; Schwarzbauer 1991). Fibronectin matrices,

assembled and organized into fibrils by the fibroblasts, perform several functions, including providing a provisional cell adhesive matrix for keratinocytes during wound healing and helping to organize the matrix framework (reviewed in (McDonald 1988)).

The dermis, especially the papillary dermis, is perfused with numerous capillary blood vessel loops branching off subcutaneous arteries, and supplying the dermis and epidermis. The capillaries form a plexus (or network) which is oriented parallel to the epidermis (Odland 1991).

Numerous myelinated and unmyelinated nerves, also organized into plexuses, provide the skin with the ability to respond to the main stimuli of heat, cold, pain, and touch (Odland 1991). Nerves penetrate the basement membrane and reach the basal layer of keratinocytes, forming associations with the Merkel cells (Sinclair 1973).

The main cellular component of the dermis is the dermal fibroblast. Fibroblasts synthesize, degrade, organize, and remodel the dermal matrix, and contribute to the basement membrane (Odland 1991). During wound healing, the fibroblasts are responsible for contracting the wound to speed healing, and remodelling the dermis (Clark 1989; Fukamizu and Grinnell 1990; Klein *et al*, 1991; Schiro *et al*, 1991).

1.3 The basement membrane

The basement membrane, also known as the basal lamina, is a thin layer of proteins that separates the dermis and epidermis. The basement membrane provides an attachment substrate for the keratinocytes of the epidermis, and is probably involved in maintaining the basal cell phenotype (see below).

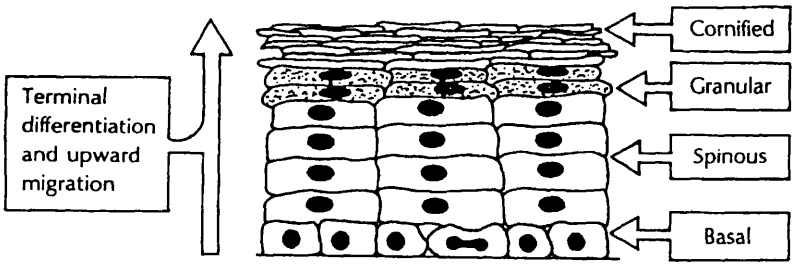
The basement membrane has two regions, recognized and named by their appearance in transmission electron microscopy (TEM). The *lamina lucida*, closest to the epidermal cells, appears as an empty space on TEM, while the *lamina densa*, named for its dense appearance, lies below.

The basement membrane is composed of a number of proteins, but primarily consists of collagen type IV and laminin (reviewed in (Timpl 1989)). Collagen type IV and laminin bind to each other and form a meshwork, which incorporates other proteins of the basement membrane, such as nidogen and heparan sulfate proteoglycans. Collagen type VII, which connects epidermal cells through their hemidesmosomes to the dermis, spans the basement membrane (Ellison and Garrod 1984; Lane *et al*, 1985). Loss of anchoring filament proteins located in the basement membrane (epiligrin/kalinin/nicein) may be responsible for junctional epidermolysis bullosa, a blistering disease in which the epidermis detaches from the basement membrane (reviewed in (Uitto and Christiano 1992)). The basement membrane does not serve simply as an adhesive substrate, but can also convey information to the epithelial cells (discussed below and in Chapters 3 and 4).

1.4 The epidermis

The epidermis is a stratified epithelium consisting primarily of keratinocytes (Matoltsy 1960; Lane *et al*, 1985; Watt 1988; Watt 1989; Fuchs 1990; Odland 1991). Mature epidermis has a relatively uncomplicated structure, consisting of 10-20 layers (including cornified layers), depending on body site (Fig. 1). The degree of terminal differentiation (terminal because the keratinocytes can no longer divide) is indicated by the spatial location of the keratinocyte. Thus, basal cells are not terminally differentiated, and stratum corneum cells, at the outer surface of the epidermis, are at the endpoint of terminal differentiation. Proliferation is largely confined to the basal layer of keratinocytes (also known as the *stratum germinativum*) that adheres to the basement membrane. Stem cells, which have been proposed to exist in the epidermis, would reside in the basal layer (Bartek *et al*, 1985; Hall and Watt 1989; Watt 1989) and may be present in hair follicles (Cotsarelis *et al*, 1990). Evidence for the presence of stem cells in the epidermis includes the observation that only about 10% of the basal keratinocytes can proliferate and form colonies after irradiation (Withers 1967; Potten and Hendry 1973). *In vitro*, stem cells are not lost, suggested by the ability of cultured keratinocytes to persist for years when grafted onto a

Figure 1. Schematic illustration of epidermis. Taken from (Watt 1989), with permission of the publisher.


Terminal differentiation and upward migration
Cornified
Granular
Spinous
Basal

patient (Gallico *et al*, 1984), and the presence of a sub-population which can form large colonies (Barrandon and Green 1987b). There is some evidence (e.g. limited proliferation of a subpopulation in culture (Barrandon and Green 1987b)) that daughter cells undergo several additional rounds of mitosis (forming the "transit amplifying" population) before down-regulating proliferation (discussed in (Watt 1988; Hall and Watt 1989)).

On an as yet unidentified signal, committed cells downregulate adhesion and leave the basal layer, initiating the program of terminal differentiation ((Adams and Watt 1989; Adams and Watt 1990) and discussed below). Although basal keratinocytes are already differentiated in the sense that they do not produce any other cell type, differentiation here is generally intended to mean the sequence of a stem cell in the basal layer producing a daughter cell which ultimately terminally differentiates (Watt 1988). As keratinocytes move through the upper layers, they increase in size and go through a defined sequence of differentiation, moving through the granular layers (*stratum granulosum* or spinous layers) and ultimately forming cornified envelopes (the *stratum corneum*), anuclear cell skeletons with a tough protein envelope filled with keratins, which form the outermost layer of the epidermis (reviewed by (Matoltsy 1986)). Keratinocyte intercellular lipids, released by fusion of lamellar granules with the plasma membrane (Odland 1960), take the place of desmosomes in maintaining the epidermal barrier in cornified cells (Swartzendruber *et al*, 1989; Wertz and Downing 1991) and undergo biochemical changes in composition during differentiation (reviewed by (Watt 1989)). As the cornified cells are shed from the epidermis, basal cells are continuously proliferating to maintain the differentiated layers. The average length of time for a basal cell to reach the outer layer of the epidermis is about 40-56 days (Halprin 1972).

1.4.1 Epidermal adnexae

The epidermis has a number of associated structures, or adnexae: hair follicles and two types of sweat glands (reviewed in (Odland 1991)). Hair follicles cover the body, at varying densities, except for the palms and soles. Hair consists of keratinous fibers, growing out of epithelial hair

follicles embedded in the dermis and continuous with the epidermis. Hair follicles undergo a recurring cycle consisting of three stages: anagen (active hair growth), catagen (follicle regression), and telogen (rest). During the anagen phase, the hair follicle extends 1-3mm into the dermis. The lowermost portion of the hair follicle expands into a bulb of epithelium, which encloses a connective tissue, or dermal, papilla. The hair itself, composed of keratin remaining from enucleated cells, is derived from the tip of the papilla and pushed upward by continued proliferation.

The hair follicle consists of several different cell type layers, surrounded by the outer root sheath keratinocytes. The main cell component of hair follicles is the follicular keratinocyte. Follicular keratinocytes differ from interfollicular keratinocytes in several features, such as constitutive expression of K6/K16, and incomplete cornification before the differentiated cells are shed (Stark *et al*, 1987; Lenoir *et al*, 1988). Hair follicles also have an associated sebaceous gland, responsible for the oily coating of the hair shaft. Stem cells may reside in the hair follicle (Cotsarelis *et al*, 1990), perhaps identifiable by K19 expression: K19-expressing keratinocytes are found in a defined region of the hair follicle, and are able to differentiate into several follicle cell types (Stasiak *et al*, 1989).

Two types of sweat glands are associated with the epidermis: eccrine and apocrine. Eccrine sweat glands are those that secrete sweat directly onto the surface of the epidermis and are present all over the body. Sweating aids thermoregulation and excretion of waste products (Sato *et al*, 1991). Three types of epithelial cells make up the sweat gland: dark (unknown function), clear (secretory), and myoepithelial (contractile; for sweat expulsion or support against hydrostatic pressure). Eccrine sweat glands respond to cholinergic stimulation. Apocrine sweat glands are those that are most abundant in areas such as the arm pit and groin; their products are secreted into the hair follicle (Odland 1991). Apocrine glands respond to α-adrenergic stimulation by contracting their myoepithelial cells.

1.5 Markers of keratinocyte terminal differentiation

Keratinocyte markers, many of which are shared with other epithelial cell types, differentiate the keratinocytes from other cell types. Involucrin, synthesized *in vivo* in the upper spinous layers, is deposited on the cytoplasmic side of the plasma membrane and assembled into the cornified envelope (Rice and Green 1977; Banks-Schlegel and Green 1981). Involucrin is a useful marker for cultured keratinocytes which have initiated terminal differentiation; immediate suprabasal cells and even some cells in low calcium monolayers express soluble involucrin (Watt and Green 1982; Watt 1983). Epidermal transglutaminase, which cross-links involucrin and makes it insoluble, is activated by a calcium influx that occurs when the cornifying cells become permeable (Thacher and Rice 1985).

Loricrin is a recently described structural protein that also contributes to cornified envelopes (Mehrel *et al*, 1990; Hohl *et al*, 1991). Unlike involucrin, loricrin is insoluble. Filaggrin is synthesized in granular cells as profilaggrin; in cornifying cells profilaggrin proteolytically processed to filaggrin is involved in packing the keratin filaments into an aggregrate (Dale *et al*, 1978; Dale *et al*, 1985).

The keratins are a family of epithelial-specific proteins whose prominence in the keratinocyte cytoskeleton is responsible for their name. Keratins are present in all keratinocytes, accounting for ~30% of the protein mass in basal keratinocytes and ~80% in cornified keratinocytes (Steinert and Idler 1975; Sun and Green 1978). There are two classes of keratins, acidic (or type I intermediate filaments) and neutral-basic (or type II), established by their migration in two-dimensional gel electrophoresis and sequence similarity; each has been assigned a number (Hanokoglu and Fuchs 1982; Moll *et al*, 1982a; Woodcock-Mitchell *et al*, 1982; Nelson and Sun 1983; Sun *et al*, 1983a; Steinert *et al*, 1984; Lane *et al*, 1985; Steinert and Freedberg 1991). Keratins are restricted in their association: only heterodimeric partners composed of type I and II can form; certain type I and type II keratins tend to be co-expressed and associate (Sun *et al*, 1984). Thus, expression of a single keratin chain (e.g. by transfection of a single functional keratin cDNA expression construct into fibroblasts (Lu and

Lane 1990)) does not result in a filament, i.e. keratins are obligate heteropolymers. All epithelial cells therefore express at least two keratins.

At present, almost 30 different keratins have been identified (Steinert and Freedberg 1991). Epidermal interfollicular keratinocytes express a subset of the keratin family: basal keratinocytes express keratins 5 and 14; suprabasal keratinocytes express keratins 1 and 10/11 (Fuchs and Green 1980; Moll *et al*, 1982b; Nelson and Sun 1983; Sun *et al*, 1983b). Palmar and plantar epidermal keratinocytes additionally express keratin 9 ((Moll *et al*, 1987) and Chapter 3, Appendix). During wound healing, hyperproliferation, cell culture, and disease, additional or mutated keratins can be detected, for example, the hyperproliferation-associated keratins 6 and 16 (Weiss *et al*, 1984; Kopan *et al*, 1987; Lane *et al*, 1992a; Pei *et al*, 1992).

Stratified, squamous keratinocytes of other body sites express keratins in addition to K5 and K14, e.g. mucous areas such as the esophagus and mouth express keratin partners 4 and 13 (Galvin *et al*, 1989). Corneal epithelial cells express keratins 3 and 12 (Galvin *et al*, 1989). "Simple", i.e. unstratified epithelia — including embryonic skin — express keratins 8 and 18 (Moll *et al*, 1982a; Moll *et al*, 1982b). It is unclear what the function of different keratins might be, but presumably there is a site-specific function.

Keratin structure, especially higher order complexes, is just beginning to be elucidated. Keratin polypeptides consist of a central α-helical rod domain, and divergent N- and C-terminal ends. Individual keratin chains associate in a parallel, in-register coiled-coil molecule through the central α helical rod domain (Steinert and Freedberg 1991). Most of the divergence between keratin polypeptides is in the N- and C-terminal end domains, which are the basis of keratin chemistry and probably function in different cells (Steinert and Freedberg 1991). Higher order structures, i.e. a 4-chain complex, probably forms in an anti-parallel conformation, either in or slightly out of register (discussed in (Steinert and Freedberg 1991)). Further complexes, forming the tonofilaments, also form; probably from 4-chain complexes arranged slightly out of register and stacked.

Differential keratin gene expression presumably has specific functions, but these have not yet been elucidated. Keratin gene regulation and expression can influenced by several factors, including retinoic acid (vitamin A; (Kopan *et al*, 1987)); hormones (Roop *et al*, 1987); the environment (Sun *et al*, 1984); and disease (reviewed in (Galvin *et al*, 1989)).

1.5.1 Keratinocyte adhesion structures and molecules

1.5.1.1 Desmosomes and hemidesmosomes

Two specialized adhesive structures, desmosomes and hemidesmosomes, are prominent in keratinocytes and contribute to the mechanical strength of skin. Desmosomes, keratinocyte cell-cell adhesive structures, are present on all but the basement membrane face of all keratinocytes, and connect to the keratin cytoskeleton, forming a continuously connected basal layer that is water-impermeable. Desmosomes consist of a plaque containing intracellular proteins, and cadherin-like glycoproteins (DGI-III (Magee and Buxton 1991; Wheeler *et al*, 1991)) which span the plasma membrane and connect to an adjacent desmosomal plaque on a neighboring cell. Epidermolysis bullosa simplex, in which a defect in the K5 or K14 protein (Coulombe *et al*, 1991; Lane *et al*, 1992a; Uitto and Christiano 1992), prevents connection to the intracellular proteins of the desmosomal plaque, results in the basal keratinocytes splitting — in the plane of the basement membrane — when mechanically stressed.

Desmosome assembly is highly dependent on extracellular calcium levels (Hennings *et al*, 1980; Watt 1984; Watt *et al*, 1984). Removal of calcium *in vitro* results in loss of assembled desmosomes in all keratinocytes (although the components remain available for assembly) (Magee *et al*, 1987). The number of desmosomes increases during the basal to spinous layer transition. During the transition from the granular to the cornified layers, there are structural alterations in the desmosomes, culminating in desmosome destruction in cornified cells.

Hemidesmosomes are not actually "half-desmosomes" (proposed in (Staehelin 1974)); they share with desmosomes the ability to bind to keratin filaments (suggested by EM), but have a distinctive structure (Jones and Green 1991). Hemidesmosomes are proposed to help bind the basal keratinocytes to the dermis through collagen type VII filaments, which span the basement membrane. The $\alpha_6\beta_4$ integrin is one of the hemidesmosomal components (Stepp *et al*, 1990; Sonnenberg *et al*, 1991), and although its function is not yet defined, it probably interacts with keratin filaments through the β_4 cytoplasmic tail. Bullous pemphigoid, a disease characterized by epidermal blistering, or detachment from the basement membrane, is caused by an autoimmune response against a hemidesmosome component, the bullous pemphigoid antigen, or BPA (reviewed in (Uitto and Christiano 1992)).

1.5.1.2 Cadherins

(see also Addendum, p. 30)

Cadherins are a family of homophilic, calcium-dependent cell-cell adhesion molecules, at least one member of which is expressed by virtually all cells (reviewed in (Takeichi 1990; Magee and Buxton 1991; Takeichi 1991)). Keratinocytes express E-cadherin in all epidermal layers ((Hirai *et al*, 1989; Nicholson *et al*, 1991; Wheelock and Jensen 1992); Chapter 3) and P-cadherin in the basal layer (Hirai *et al*, 1989; Nicholson *et al*, 1991). Cadherins have been suggested to be central to cell-sorting mechanisms and selective adhesion, allowing groups of cells to associate and form organs (Takeichi 1990; Takeichi 1991). Re-distribution of β_1 integrin, vinculin, E- and P-cadherin, and desmoplakin to cell borders is delayed by anti-E-cadherin antibody during calcium-induced stratification ((Wheelock and Jensen 1992); K. Hodivala, pers. comm.).

1.5.1.3 Integrins

The integrins are a large family of cell surface glycoproteins mediating cell-cell and cell-ECM adhesion (reviewed in (Hynes 1987; Hemler 1990; Ruoslahti 1991; Hynes 1992). Each integrin consists of one α and one β subunit (schematically illustrated in Fig. 2); although there is a large number of possible heterodimers — to date 14 α and 8 β subunits

have been described — only a restricted set of subunit associations actually occurs. Ligand specificity is largely determined by heterodimer composition, although the same integrin in different cell types can have different specificities (e.g. (Elices and Hemler 1989; Languino *et al*, 1989; Hynes 1992)). Several integrins (e.g. $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ (Hynes 1992)) recognize a 3 amino acid domain (RGD, or arginine-glycine-aspartate) found in many ECM proteins for binding.

The β_1 integrins were originally grouped together as the "VLA" antigens (α_1-$\alpha_6\beta_1$), named for their induction late in the cascade of T-cell activation (Hemler 1990). This terminology, based on the α subunit, is still in use; VLA-1 refers to $\alpha_1\beta_1$, VLA-2 is $\alpha_2\beta_1$, and so on. Based on this system, attempts were made to group other integrins into families based on the β subunit. The original grouping of integrins into families, though useful, has proved to be awkward, as it is now known that some α subunits can associate with several β subunits. For example, α_v can associate with β_1, β_3, β_5, β_6, and β_7 (Hynes 1992).

Integrins are not only receptors for ECM proteins, but can also recognize some members of the immunoglobulin (Ig) superfamily. For example, $\alpha_4\beta_1$ can bind both fibronectin and VCAM-1 (expressed on endothelial cells)(Taichman *et al*, 1991). $\alpha_L\beta_2$, a member of the leukocyte integrins (β_2 integrins, restricted to leukocytes and consisting of a common β_2 subunit with one of three α subunits (Sanchez-Madrid *et al*, 1983; Konter *et al*, 1989; Simon *et al*, 1992)) can recognize ICAM-1 and -2, also Ig members. The leukocyte integrins are typically involved in cell-cell adhesion necessary for immune interaction; they provide the best evidence to date for a cell-cell adhesion role for the integrins (Campanero *et al*, 1990; Hemler *et al*, 1990).

1.5.1.3.1 Integrin structure and cytoplasmic interactions

The ligand binding sites of the integrins are formed by both the α and β subunit. The cation binding sites, a conserved feature of α subunits, are intimately associated with the ligand binding site. The β subunit has a series of conserved cysteine-rich domains (reviewed in (Hynes 1992)).

Both the α and β subunits have short cytoplasmic tails (around 50aa, with the exception of β_4 (Kajiji *et al*, 1989; Suzuki and Naitoh 1990)); to

Addendum to 1.5.1.2 Cadherins

The conservation of cadherin amino acid sequence is greatest in the C-terminal, cytoplasmic domain (reviewed in Takeichi 1991). Mutations of this domain have no effect on homophilic binding, but prevent interaction of the cytoplasmic domain with the cytoskeleton and cadherin localization at the *zonula adherens* (at which underlying cytoplasmic plaques and associated cytoskeletal components, including actin can be localized). The catenins (α, β, and γ) interact with the cytoplasmic domain of the cadherins, perhaps mediating the cadherin-actin interaction: catenin-bound wild-type cadherins can bind globular actin, but C-terminal mutated cadherins, which cannot bind catenins, can not bind actin. Plakoglobin, a desmosome and adherens junction component (Salomon *et al*, 1992) is related to (but distinct from) the catenins (Peifer *et al*, 1992). As discussed above, the desmogleins, transmembrane components of desmosomes, have amino acid homologies to the cadherins and may mediate a similar cell-cell adhesion.

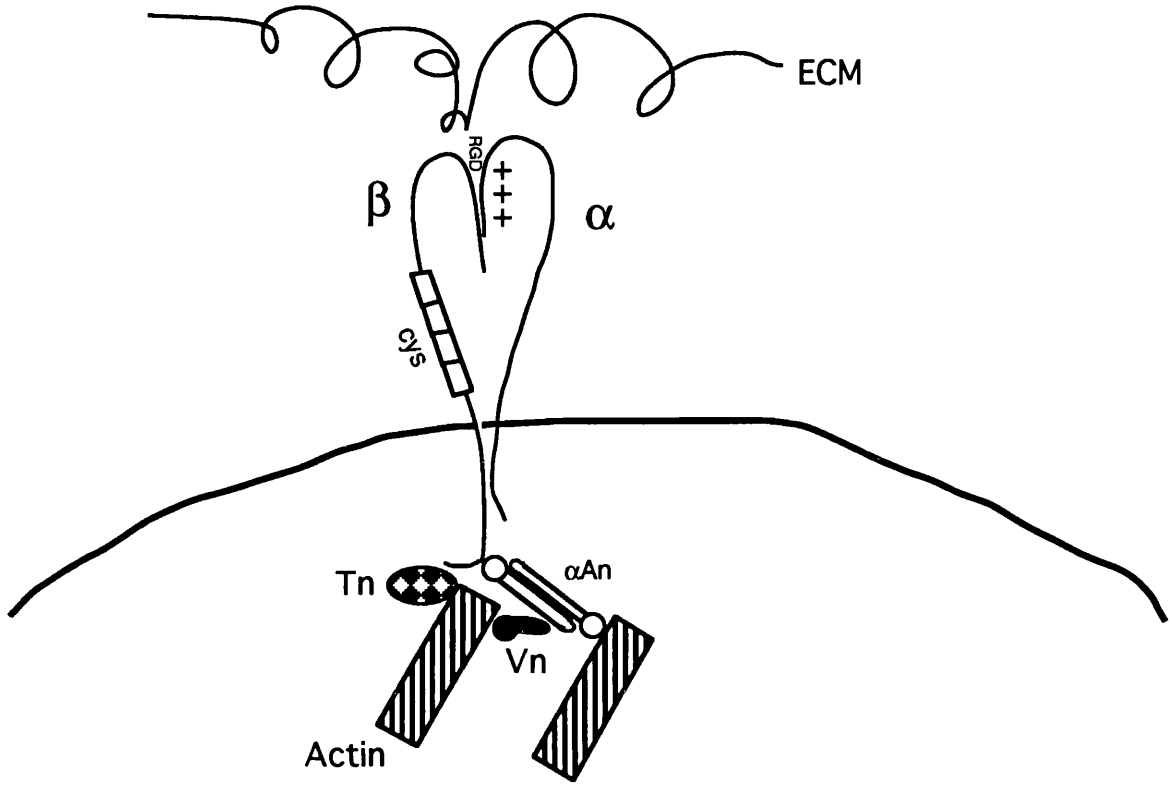
The N-terminal, extracellular domain is responsible for the homotypic specificity of the cadherins. Binding of cadherins to each other is mediated by the N-terminal 113 amino acids; specificity can in some cases be altered by mutation of just two amino acid residues.

Along with cell-sorting phenomena, the cadherins have also been suggested to be involved with the cell layer segregration characteristic of tissues such as the epidermis. Thus, as basal keratinocytes terminally differentiate, entering the suprabasal layers, P-cadherin expression is lost, while E-cadherin expression is retained.

Peifer, M., McCrea, P. D., Green, K. J., Wieschaus, E. and Gumbiner, B. M. (1992). The vertebrate adhesive junction proteins beta-catenin and plakoblobin and the Drosophila segment polarity gene armadillo form a multigene family with similar properties. *J. Cell Biol.* **118**: 681-691.

Salomon, D., Ayalon, O., Patel-King, R., Hynes, R. O. and Geiger, B. (1992). Extrajunctional distribution of N-cadherin in cultured human endothelial cells. *J. Cell Sci.* **102**: 7-17.

Figure 2 Schematic illustration of a β_1 integrin heterodimer, spanning the plasma membrane, and interacting with the ECM and the actin cytoskeleton at a focal contact. ECM, here represented by fibronectin, with an RGD sequence for integrin recognition, is shown at the binding site formed by the α and β subunits. The cysteine repeats ("cys") on the β subunit are shown, as are the cation binding sites ("+") on the α subunit. The cytoplasmic tail of the β subunit is shown interacting with talin ("Tn") and α-actinin ("αAn"), each of which interacts with actin. Vinculin ("Vn") is shown connecting actin and α-actinin. Modified from (Hynes 1992) and (Pavalko *et al*, 1991).


ECM
RGD
β
α
cys
Tn
αAn
Vn
Actin

date, only the β_1 subunit has been demonstrated to interact directly with the cytoskeleton, although the α subunit may have a role in focal contact localization (see below).

The integrins were originally named for their ability to "integrate" the extracellular matrix and the cytoskeleton. In cultured cells, integrins are often localized in focal contacts, areas of close cellular contact with the substrate (reviewed in (Burridge and Fath 1989; Pavalko *et al*, 1991; Turner and Burridge 1991)).

1.5.1.3.2 Keratinocyte integrins

There is growing evidence that extracellular matrix (ECM) receptors of the integrin family play an important role in maintaining the spatial organization of the different keratinocyte layers. The major integrins expressed by keratinocytes in culture (identified by immunofluorescence staining and immunoprecipitation) are $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_4$ (Adams and Watt 1990; Carter *et al*, 1990; De Luca *et al*, 1990; Staquet *et al*, 1990; Adams and Watt 1991). Expression of each integrin both in culture and in the epidermis is confined to the basal layer (Fradet *et al*, 1984; Sonnenberg *et al*, 1986; Wayner *et al*, 1988; De Strooper *et al*, 1989; Peltonen *et al*, 1989; De Luca *et al*, 1990; Adams and Watt 1991; Nicholson and Watt 1991), as identified by immunofluorescence staining. The decrease in integrin protein expression during terminal differentiation is correlated with a decrease in the subunit mRNAs; this has been demonstrated by Northern blotting of cultured keratinocytes (Nicholson and Watt 1991; Hotchin and Watt 1992) and by *in situ* hybridization of epidermis (Chapter 4). The decrease in steady-state mRNA levels of α_5 and β_1 is a result of inhibition of transcription of the α_5 and β_1 genes during terminal differentiation (Hotchin and Watt 1992).

α_1, expressed in low amounts in cultured keratinocytes (Belkin *et al*, 1990; Adams and Watt 1991) has been reported to be weakly expressed (Buck *et al*, 1990; Hertle *et al*, 1991) or absent (Hemler *et al*, 1984; De Luca *et al*, 1990; Nazzaro *et al*, 1990; Zambruno *et al*, 1991b) *in vivo*. α_v is expressed in cultured keratinocytes, forming a heterodimer with β_5 (Adams and Watt 1991; Marchisio *et al*, 1991), but *in vivo* is weakly

expressed (Nazzaro *et al*, 1990; Hertle *et al*, 1991). α_5 is strongly expressed in cultured keratinocytes several days after isolation from epidermis (Takashima and Grinnell 1985; Toda *et al*, 1987), or if isolated from healing cutaneous wounds (Grinnell *et al*, 1987; Grinnell 1990), but *in vivo* is weakly expressed (Wayner *et al*, 1988; Hertle *et al*, 1991) or not detected by some investigators (Peltonen *et al*, 1989; Nazzaro *et al*, 1990; Pellegrini *et al*, 1992). Possible keratinocyte expression of other integrins is discussed in Chapter 3.

Cell adhesion assays have been used to determine the substrate to which keratinocyte integrins bind. $\alpha_5\beta_1$ binds the RGD sequence in the fibronectin central cell binding domain (Adams and Watt 1990; Carter *et al*, 1990; Adams and Watt 1991). $\alpha_2\beta_1$ is a receptor for collagens I and IV (Carter *et al*, 1990; Staquet *et al*, 1990; Adams and Watt 1991) and also has a role in adhesion to laminin (Carter *et al*, 1990; Adams and Watt 1991). $\alpha_3\beta_1$ is a laminin receptor (Carter *et al*, 1990; Adams and Watt 1991) and also binds the recently described basement membrane component epiligrin (Carter *et al*, 1991), which is probably identical to kalinin (Rousselle *et al*, 1991; Watt and Hotchin 1992). In keratinocytes, $\alpha_3\beta_1$ can also bind fibronectin (Carter *et al*, 1990; Staquet *et al*, 1990) (although $\alpha_5\beta_1$ appears to be the major, high affinity receptor for fibronectin (Adams and Watt 1990)) and collagen (Staquet *et al*, 1990). $\alpha_v\beta_5$ mediates keratinocyte adhesion to vitronectin (Adams and Watt 1991). Although $\alpha_6\beta_4$ has been reported to bind laminin in cell adhesion assays (De Luca *et al*, 1990), this has not been confirmed (Adams and Watt 1991). The ligand may be a keratinocyte-specific ECM protein (Carter *et al*, 1990). $\alpha_6\beta_4$, which participates in hemidesmosomes (Kurpakus *et al*, 1990; Stepp *et al*, 1990; Sonnenberg *et al*, 1991) is discussed above.

In cultured keratinocytes, some integrins can be localized to focal contacts — areas of close association with the substrate, into which actin filaments terminate — with several proteins at the cytoplasmic face of the focal contacts. The focal contact protein distribution in keratinocytes (vinculin, talin, actin, and α-actinin) has been documented (Kubler *et al*, 1991). β_1 has been demonstrated to bind directly to vinculin and α-actinin through its cytoplasmic domain (Turner and Burridge 1991). There is some controversy about which integrin heterodimers are localized to focal contacts, but this may be due to the substrate on which the keratinocytes

adhere (Carter *et al*, 1990), whether they are migrating or stationary (Marchisio *et al*, 1991), and whether calcium-dependent intercellular junctions are present (Carter *et al*, 1990; Larjava *et al*, 1990; Marchisio *et al*, 1991). Thus, on a pure fibronectin substrate, $\alpha_5\beta_1$ is found in focal contacts, on a collagen substrate, $\alpha_2\beta_1$, and on a laminin substrate $\alpha_3\beta_1$ (Carter *et al*, 1990). In keratinocytes spread on collagen, β_1 integrins and vinculin can be co-localized (Guo *et al*, 1990).

Keratinocyte integrins are not only found in focal contacts, but also are diffusely distributed over the entire cell surface and appear to be concentrated at cell-cell juntions. This has led to the proposal that besides mediating adhesion to ECM, the integrins may have a cell-cell adhesion role (Kaufmann *et al*, 1989; Carter *et al*, 1990; Larjava *et al*, 1990; Marchisio *et al*, 1991). In low calcium medium, integrins are diffusely distributed around the cell membrane. However, when stratification is induced by addition of calcium, α_2, α_3, and β_1 become concentrated at cell-cell boundaries (Carter *et al*, 1990; Larjava *et al*, 1990; Marchisio *et al*, 1991).

The strongest evidence for integrin cell-cell adhesion comes from antibody disruption of cell-cell contacts. In both low calcium (Larjava *et al*, 1990) and standard calcium medium (Carter *et al*, 1990), intercellular contacts can be disrupted by an anti-β_1 antibody. However, if the integrins are functioning as cell-cell adhesion molecules, there is no demonstrated ligand — for example, ECM proteins do not accumulate at cell-cell junctions (Larjava *et al*, 1990) — and no direct integrin-integrin interaction has been demonstrated. Also, Tenchini *et al* (Tenchini *et al*, 1993) have shown that when keratinocytes are allowed to form calcium-dependent cell-cell contacts in the absence of an adhesive substrate, no integrin monoclonal antibodies tested were able to block cell-cell adhesion.

Integrins also have a role in keratinocyte migration; this is discussed in Chapter 4.

During terminal differentiation keratinocytes lose adhesiveness to fibronectin, laminin and collagen type IV; the adhesive changes precede loss of the β_1 integrins from the cell surface (Adams and Watt 1990). There is evidence that a decrease in the ligand binding ability of the $\alpha_5\beta_1$ integrin occurs in basal cells on commitment to terminal differentiation; such

down-regulation of integrin function would provide one mechanism for the selective migration of committed keratinocytes out of the basal layer (Adams and Watt 1990). The reduction in fibronectin binding is due to modulation of pre-existing receptors on the cell surface, rather than synthesis of modified receptor (Hotchin and Watt 1992). Loss or reduction of cell-substratum adhesion is one signal for keratinocyte terminal differentiation (Watt *et al*, 1988) and integrin-mediated interaction with the extracellular matrix may play a role in regulating the initiation of terminal differentiation (Adams and Watt 1989).

1.6 Aims of the project

In vitro evidence indicates that the integrins are important in regulating keratinocyte adhesion and terminal differentiation *in vitro*. I wanted to examine integrin expression *in vivo* to see if there were changes in expression associated with the epidermal morphogenesis during development and wound healing. I followed up the *in vivo* observation by finding *in vitro* models to study the significance of these changes.

Chapter 2

Materials and Methods

2.1 General notes

Sources of reagents are identified at first citation; if unidentified the supplier was Sigma Chemical Co., Poole, UK. Abbreviations are defined at first use.

2.2 Tissue handling and cell culture

2.2.1 Buffers and media used

Buffers and media made by ICRF Central Cell Services are identified as (ICRF).

2.2.1.1 Phosphate-buffered saline (PBS)

PBS (PBSA at ICRF) consisted of 8g NaCl, 0.25 KCl, 1.43g Na_2HPO_4, and 0.25g KH_2PO_4 in distilled water, titrated to pH 7.2, and autoclaved. PBSABC consisted of PBS supplemented to 1mM $CaCl_2$ and 1mM $MgCl_2$.

2.2.1.2 Hank's buffered saline solution (HBSS)

HBSS (ICRF) consisted of 8g NaCl, 0.4g KCl, 0.06g KH_2PO_4, 0.14g $CaCl_2$, 0.1g $MgSO_4$, 0.048g Na_2HPO_4, and 0.02g phenol red per liter, sterilized by 0.22μm filtration.

2.2.1.3 Versene

Versene (ICRF), a calcium and magnesium-free buffered solution containing EDTA, consisted of 8g NaCl, 0.2g KCl, 1.15g Na_2HPO_4, 0.2g Na_2EDTA, and 1.5ml 10% phenol red per liter.

2.2.1.4 Trypsin

Trypsin (ICRF), used to detach and dissociate cells, was made at 0.25% w/v in versene and used at 0.05% final concentration, i.e. diluted 1:5 in versene.

2.2.2 Collection and processing of tissue samples

2.2.2.1 Foreskin

Control tissue (normal foreskin, 0-4 year-old patients) was obtained from Dr. Herbert Barrie, Charing Cross Hospital and transported moist at room temperature. Connective tissue and mucous membranes were trimmed off and ~3mm x 7mm rectangles of tissue were frozen as described above.

2.2.2.2 Fetal skin

Fetal skin was obtained from elective abortions, through Dr. Leslie Wong, MRC Tissue Bank, Royal Marsden Hospital, as approved by the ICRF Ethical Committee. All tissues were collected and kept at 4°C or on ice and transported to the laboratory as quickly as possible, generally within 4 hours of collection. Fetal limbs were submerged in Hank's buffered saline solution (HBSS) and the skin (arm or leg and palm or sole; including some underlying connective tissue) was removed under a dissecting microscope using micro scissors and forceps.

2.2.2.3 Suction blisters

Sets of five suction blisters, 4-5 mm in diameter, were induced on hairy thigh or upper arm skin of human volunteers by the application of gentle suction (600mm Hg) for 1-2 hours until a clear, blood-free blister was formed, as described by (Kiistala and Mustakallio 1967). 6mm skin punch biopsies (including some subcutaneous fatty tissue) were taken under xylocaine local anesthetic at 1, 2, 3, 4, 6, 7, and 14 days and frozen as detailed above. Biopsies from a total of 11 volunteers were examined. The biopsies were taken by I.M. Leigh and colleagues, with prior approval from the Royal London Hospital Ethical Committee.

2.2.2.4 Psoriatic tissue

Biopsies of involved psoriatic skin were generously provided by Dr. Irene Leigh, and obtained from patients with informed consent.

2.2.2.5 Cytokine injections

Tissue from volunteers who had been injected with cytokines were generously provided by Dr. Richard Groves, Dept of Dermatology, Guy's Hospital, London (TNF-α and IFN-γ) and Dr. Richard Camp, Section of Dermatology, University of Leicester (IL-1). Volunteers were intradermally injected with 30μg of IFN-γ (Groves *et al*, 1991), and biopsies were taken at 24 and 48 hours, and 5 days. 100U TNF-α was injected and biopsies were taken at approximately 24 hours and 5 days.

As recombinant IL-1 was not available at the time, IL-1 was isolated by processing aqueous extracts of heel stratum corneum by successive ultrafiltration, reversed phase HPLC, then anion exchange HPLC to obtain a purified IL-1 fraction (Camp *et al*, 1990). Skin contains large amounts of bioactive pre-formed IL-1α, but little or no IL-1β. Material corresponding to 60 Units was injected back into the forearm skin of the corresponding original stratum corneum donor, and punch biopsied after 4 hours.

2.2.2.6 Tissue freezing

All tissues were frozen by coating in cold OCT compound (BDH Ltd, Poole, UK), placing on a cork disk, and slowly submerging in an isopentane bath cooled to approximately -70°C in liquid nitrogen. Tissues were left submerged 1-2 minutes to allow complete freezing. Tissue blocks were stored at -70°C until sectioned.

2.2.3 Tissue sectioning

All sectioning was performed by the ICRF Histopathology Unit. Blocks were mounted and sectioned at -20°C; 6μm sections were cut and

mounted on 4-well glass slides, which had been treated with aminoalkylsilane ("silane"; (Rentrop *et al*, 1986)) and stored at -70°C until use. Representative sections were stained with hematoxylin and eosin for morphological analysis.

2.2.4 Fetal tissue preparation for culture and recombination

These methods were as demonstrated by Chris Fisher (and described in (Fisher and Holbrook 1987)). Skin was isolated as detailed above and transferred to a new dish containing cold HBSS. Excess connective tissue was trimmed from the underside of the skin and discarded. Skin was cut into approximately 20mm squares, with clean edges, and transferred in a droplet of HBSS (to prevent tissue damage) to fresh cold HBSS. Skin was then either placed into culture (below) or placed in dispase to dissociate the epidermis and dermis (below).

2.2.4.1 Tissue recombinants

$20mm^2$ pieces of skin were placed into a 35mm dish with 1-2ml of dispase (a protease; Collaborative Research, Bedford, MA) at 15U/ml solution diluted in serum-free Dulbecco's modification of Eagle medium (DME) and placed at 4° C overnight, or at 37° C for one hour. The dermis and epidermis were teased apart, placed into organ culture medium using a Pasteur pipette, and then recombined as appropriate: dermis was placed into the organ culture dish on a membrane (see below), connective tissue side down. Epidermis in the correct orientation was pipetted onto the dermis and gently manipulated into place. Recombinants were then cultured as for unrecombined skin.

2.2.4.2 Fetal skin organ culture

Cultures were set up in 60mm Falcon 3037 (Becton Dickinson UK, Oxford) organ culture dishes as follows. An autoclaved, fine mesh stainless steel grid cut into a triangle was placed over the central well of the dish. A 13mm Millipore HA (nylon) 0.45µm membrane (Millipore Corp., Bedford, MA) was placed onto the grid and saturated with a drop of organ culture

medium. Skin was placed onto the membrane, dermis side down and gently pressed with fine forceps to bond the tissue to the membrane. Organ culture medium was pipetted into the central well until it just reached the membrane. Any excess medium on the membrane was removed so that the tissue received medium only through the filter, and was in contact with air on the epidermal surface. Cultures were maintained in a humidified 5% CO_2/air atmosphere and medium was changed every 2-3 days.

2.2.4.3 Organ culture medium

Medium consisted of DME, containing 100µg/ml streptomycin and 100 units/ml penicillin supplemented with 10% fetal calf serum. Ascorbate in PBS was prepared and added immediately before use to a final concentration of 300µg/ml. Alternatively, keratinocyte culture medium, supplemented with ascorbate, was also used.

2.2.5 FAD medium

FAD medium (Imperial Labs, UK; prepared by ICRF) consisted of 1/4 Ham's F12 and 3/4 DME, supplemented with 100IU/l penicillin (Gibco), 100µg/l streptomycin (Gibco), 1.8×10^{-4}M adenine, and filter sterilized (Rheinwald 1989). Before use, FAD was supplemented with "HICE", consisting of 0.5µg/ml hydrocortisone (Calbiochem), 5µg/ml insulin, 10^{-10}M cholera enterotoxin (ICN, High Wycombe, UK), 10ng/ml epidermal growth factor (Chiron Corp., Emeryville, CA, or Austral Biologics, San Ramon, CA), and 10% batch-tested fetal calf serum (FCS; Sera-Lab, Crawley Down, UK). Complete medium not used within one week was discarded.

2.2.6 Chelex treatment of serum to remove calcium and magnesium

Chelex 100 resin (100-200 mesh, sodium form; Bio-Rad, Richmond, CA), used as described in (Brennan *et al*, 1982) was swollen at 40g/l in distilled water, titrated to pH 7.4, and filtered through Whatman #1. Serum was treated by adding 20g swollen resin to 50ml FCS and stirring at room

temperature for 3 hours. Resin was removed and FCS sterilized by filtering through an 0.2μm filter. Chelexed FCS was stored at -20 °C.

2.2.7 Removal of lipids from serum

To remove retinoic acid from serum, which has been shown to have effects both on the differentiation of keratinocytes (Kopan *et al*, 1987; Choi and Fuchs 1990) and on the integrin localization in raft cultures (Asselineau *et al*, 1989), the method described by (Albutt 1966; Rothblat *et al*, 1976) to remove all lipids was followed. 450ml acetone and 450ml 100% ethanol were cooled in an ice bath and 100ml FCS was added. A white protein precipitate immediately formed. The mixture was swirled and allowed to stand in an ice bath for four hours, with occasional swirling. The delipidized protein was collected by pouring 500ml of the slurry per Whatman #1 filter placed in an 18cm Buchner funnel. A thin film of protein was attempted; thicker portions did not dry well and adhered to the filter paper. Before the solvent had cleared the precipitate, ice-cold ethyl ether (125ml per filter) was added and suction maintained for 20 minutes. The filters with precipitated protein were dried at 37°C for 30 minutes and the protein was teased off the filter paper. The protein was lyophilized overnight to remove residual water and ether, and the resulting protein was weighed and stored desiccated at 4°C. The starting protein concentration of the serum was determined using the Bradford assay (below) and the delipidized protein added to the same concentration as the original FCS to medium, which was then filtered.

2.2.8 Epidermal keratinocyte cell culture

2.2.8.1 3T3 Feeder cell layer routine culture

3T3 fibroblasts, clone J2, were routinely cultured as stocks to prepare as mitotically inactivated feeder cells, as detailed in (Rheinwald and Green 1975). J2 were cultured in DME + 10% donor calf serum (DCS) and subcultured at 1:10 every 4-5 days. Cultures were discarded when the J2 started to morphologically transform (generally within about 3 months) and fresh cells were thawed.

2.2.8.2 Preparation of J2 feeder layers

Confluent J2 were mitotically inactivated to act as feeder layers by 2-3 hour incubation in 4μg/ml mitomycin C, washed twice in versene, and trypsinized (0.05% trypsin (DIFCO) final). Feeders were seeded at 1:3 for subsequent seeding with keratinocytes. Mitotically inactivated feeders were kept up to 2 days before use. For long term storage, cells were frozen in DME + 10% DCS + 10% sterile DMSO at -70°C in an insulated container to control the rate of freezing, and then transferred to liquid nitrogen.

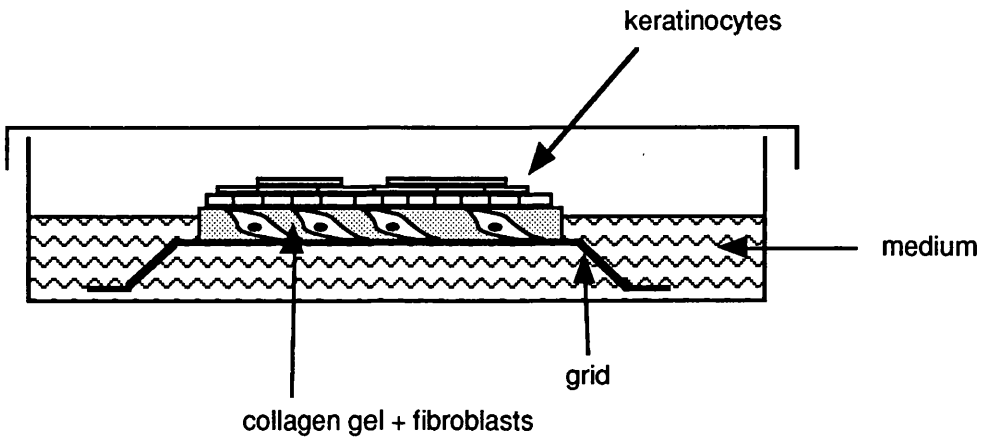
2.2.8.3 Keratinocyte subculture

Keratinocytes were cultured and passaged as originally described by (Rheinwald and Green 1975). Frozen second passage keratinocytes, derived by enzymatic dissociation of human foreskin, were seeded onto mitotically inactived J2 feeder cells at 2×10^5 on T25 flasks (T25 = rectangular tissue culture-treated polystyrene flask with a 25 cm^2 area; Falcon). Medium, designated FAD + FCS + HICE (complete medium; described above), was changed three times a week. Small colonies generally appeared within 3 days, and cultures were confluent within 7-10 days. Cultures were passaged just before or at confluence, for optimum colony forming efficiency. First the cultures were incubated in versene to detach any remaining feeders, leaving the keratinocytes attached. Suspended J2 were aspirated off, and fresh versene containing trypsin at 0.05% was added. With vigorous pipetting, a single cell suspension could be obtained which was diluted in complete medium to inactivate trypsin. Cells were pelleted at 1000rpm for 5 minutes and resuspended in complete medium. 1×10^5 cells were added per T25, or 3×10^5 per T75. Keratinocytes were frozen at 1×10^6/ml in 90% FCS/10% sterile DMSO, as described for J2 above.

2.2.8.4 Keratinocyte raft culture

2.2.8.4.1 Vitrogen/3T3 cultures

This method is based on several published methods (Bell *et al*, 1981; Asselineau and Prunieras 1984; Kopan *et al*, 1987; Asselineau *et al*, 1989); a typical culture is shown below. The main modifications included the use of Vitrogen (Collagen Corp., Fremont, CA), a denatured bovine dermal collagen, instead of rat tail collagen, and the use of 3T3-J2 fibroblasts as feeder cells, instead of human primary fibroblasts. Throughout preparation, all components except for the J2 were kept on ice to prevent polymerization of the collagen. Gels were typically cast in a volume of 2ml in 35mm diameter wells. Collagen was diluted from stock (2.8-3.3mg/ml) with 0.012M HCl, and used at 2.34mg/ml final concentration. 14ml of collagen/cell solution, sufficient for 6-35mm gels, consisted of the following: 11.2ml 2.34mg/ml collagen, 350µl 0.1M NaOH, 350µl 4.4% $NaHCO_3$, 1.4ml 10X DME (serum-free), and 700µl mitomycin C-treated J2 at 7.5 x 10^6/ml. This was pipetted gently, avoiding air bubbles, into wells and put into an incubator for at least 45 minutes, until the gels set. 2ml 1X DME + 20% DCS was added to equilibrate the gel to 10% serum and gels were kept in the incubator until seeded with keratinocytes (immediately to one day). Gels were seeded with 1x 10^5 pre-confluent keratinocytes per 35mm well and fed as for ordinary keratinocyte cultures. Cultures were generally confluent within 7 days. To raise cultures to an air/medium interface, they were released from the edge of the dish and transferred to stainless steel grids, elevated about 2mm above the surface of the dish. Medium was added, making sure the top of the gel was not covered, and cultures were maintained for 1-2 weeks.



2.2.8.4.2 Rat tail collagen cultures

2.2.8.4.2.1 Prototype method

To follow published methods, and increase quality of the cultures (see, for example (Brennan *et al*, 1982)), rat tail-derived collagen (RTC) was also used, seeded with either mitotically-inactivated J2 or human dermal fibroblasts (passage 7-9). RTC was used at approximately 2.4mg/ml, brought to physiological medium concentration with 10X DME and titrated with 1M NaOH to the correct pH. Cells were added as above and cultured.

2.2.8.4.2.2 Kaur method

To improve the morphology and consistency of the gels, a method developed by Pritinder Kaur, Hanson Center for Cancer Research, Adelaide, Australia (personal communication) and based on (Kopan *et al*, 1987) was followed. Each raft culture consisted of 4ml collagen mixture seeded with 1.5×10^5/ml human dermal fibroblasts (derived from foreskin) or J2 as appropriate. Rat tail collagen (Collaborative Research #40236) was diluted to 3.82 mg/ml in 0.02M acetic acid. Neutralization was with "reconstitution buffer", consisting of 2.2g $NaHCO_3$ and 4.77g Hepes in 100ml 0.05M NaOH. To pour four rafts, 12.6 ml collagen, 2ml reconstitution buffer, 4ml 5X DME medium (with 5X HICE), and 2ml FCS containing 3×10^6 fibroblasts was mixed well, on ice, then poured into 35mm non-tissue culture treated petri dishes (Falcon). Gels formed within one hour at 37° C. Each raft was then seeded with 10^6 keratinocytes in 2ml FAD +HICE +FCS and keratinocytes were allowed to attach and grow for four days, by which time confluence was reached and gels had contracted. They were then raised as described above.

2.2.8.4.3 Cellagen inserts

Plastic 13mm diameter culture dish inserts, holding a collagen-coated membrane (ICN) were also used, as they offered more convenience and reproducibility, and allowed separation of feeder and keratinocyte cells during co-culture. Feeder cells (either mitotically-inactivated J2 or human dermal fibroblasts (passage 7-9)) were seeded onto the underside of the culture insert, allowed to attach, and the insert was flipped so that keratinocytes (0.33×10^5) could be seeded into the wells. These were cultured as collagen rafts, above.

2.2.8.5 Cytokine treatment

Keratinocyte cultures were treated with cytokines as indicated in Chapter 5, in either normal FAD + 10% FCS + HICE, or in FAD + 0.5% FCS, for either 24 or 48 hours. Human recombinant interferon-γ was a generous gift of Dr. I. Kerr, ICRF. Human transforming growth factor-β_1 was obtained from the National Reference Standards Laboratory, South Mimms, Herts, UK, or from Sigma Chemical Co. Human interleukin-1β, interleukin-6, and interleukin-8 were obtained from the National Reference Standards Laboratory. Human recombinant tumor necrosis factor-α was obtained from the National Reference Standards Laboratory, and also was a gift from F. Balkwill, ICRF. Cytokines were stored in accordance with the manufacturer's instructions (usually at -70°C) in single-use aliquots.

2.2.9 Embryo stem cell culture

2.2.9.1 STO cell culture

STO cells are a mouse embryo-derived feeder layer for ES cell culture (Robertson 1987) and were a kind gift of Rosa Beddington, ICRF. Cells were subcultured at 1:10 in DME + 10% DCS, usually reaching confluence within 5 days. When the cells started overgrowing or when they appeared to be making poor feeder layers, they were discarded and a fresh vial thawed. Cells were frozen for storage, using the cells from half a confluent

T75 per 1ml per vial. Cells were resuspended in half the total volume required in DME + 10% serum and the total made up by adding an equal volume of freezing medium: 60% DME + 10% DCS; 20% FCS; and 20% DMSO.

2.2.9.2 Preparation of STO feeder layers

Feeder cells were mitotically inactived by either mitomycin C treatment or X-irrradiation. X-irradiation was generally used, as it was more effective at preventing feeders dividing. 1-2 T75 flasks were X-irradiated at a distance of 14 inches, for 10 minutes at 10mA at 320kV (total dose was 6000 rads). Mitotically inactivated feeders were plated onto 0.1% sterile porcine gelatin-coated (300 bloom; BDH) flasks at 5×10^4 cells/cm^2. Treated feeders were kept for up to 5 days before use.

STO feeders cannot survive in HAT medium, whereas ES cells can (R. Beddington, pers. comm.). On one occasion, the STO cells were not mitotically inactivated by mitomycin C and they proliferated, competing with the ES cells for medium and space. Several passages in HAT medium were sufficient to eliminate STO. Subsequently, only X-irradiation was used.

2.2.9.3 Embryo stem (ES) cell culture

2.2.9.3.1 Medium preparation

ES medium consisted of DME supplemented with 20% batch-tested FCS, 1X non-essential amino acids (Gibco-BRL), and 0.1 mM β-mercaptoethanol (Gibco-BRL).

2.2.9.3.2 ES culture

Primary ES cells, derived from 129 (GPI 1C) strain mouse blastocysts according to Robertson (Robertson 1987) were obtained as a frozen stock from Rosa Beddington, ICRF and designated CCE and CC3. The original

vials were thawed and plated onto STO feeders prepared as detailed above. Once established, the ES cells grew rapidly from single cells to colonies which covered the flask within 2-3 days. Cultures were fed on the second and subsequent days. Flasks in which a significant number of cells had started to differentiate (e.g. spherical aggregrates formed and large endoderm cells were evident at margins of colonies) were discarded. Spontaneous differentiation was minimized by frequent passaging.

2.2.9.3.2.1 Subculture

Flasks were fed 2-3 hours before passaging, to improve viability. A minimum of ten T25 flasks of STO feeders per T25 of ES to passage were prepared. Each flask was washed once with warmed Versene (5ml for a T25). 4ml Versene and 1.5ml 0.25% freshly thawed trypsin were added to each flask and returned to the incubator for 3 minutes.

The cell sheets were gently dislodged by rocking the flasks; once the sheets had started peeling off, the cells were vigorously pipetted with a plugged Pasteur pipette to break up the cell clumps (at least 10 times). Flasks were returned to the incubator for 2-3 minutes. The cell suspension was pipetted until it was relatively free of aggregrates. Carrying over aggregrates yielded a poor, differentiated culture. Cell suspensions were spun down in ES medium and resuspended at 1 x 10^7/ml. Cells were plated at approximately 2 x 10^6 cells per T25 flask.

2.2.9.3.3 Freezing and thawing

ES cells were frozen at 5 x 10^6 cells per 1ml vial as follows. To cell suspensions at 1 x 10^7/ml, an equal volume of 2X freezing medium was added (consisting of 60% complete ES medium, 20% FCS, and 20% DMSO). Vials were frozen and thawed as detailed for keratinocytes.

2.3 Indirect immunofluorescence staining

The same basic method was used for all antibodies, after testing this and other staining protocols. Throughout staining, PBS with calcium and magnesium salts added (PBSABC) was used; the cations may be necessary for the integrins to be in the correct conformation for antibody recognition (Hemler 1990). Blocking, antibody dilution/incubation, and washing were all carried out in PBSABC + 0.1% bovine serum albumin (BSA, fraction V, Ig-free) + 0.02% Triton X-100 + 0.02% sodium azide, designated PBS/BSA, to minimize background fluorescence through nonspecific antibody binding (Howlett *et al*, 1987).

Sections were removed from the -70° C freezer, allowed to warm for several minutes, and rehydrated in PBSABC. Sections were fixed for 30 minutes in 4% freshly diluted formaldehyde (BDH) in PBSABC. Reactive aldehyde groups were blocked by rinsing in 0.1M glycine in PBSABC, followed by washing 3x over 5 minutes in 0.1M glycine. Slides were rinsed in PBS/BSA, followed by incubation in PBS/BSA for at least 30 minutes, with one change.

Slides were transferred to a large, lidded tray humidified with PBS-soaked tissues. Antibody against the antigen of interest was diluted to working strength in PBS/BSA and 20µl applied per well. Antibodies were titered first on foreskin sections for optimum dilution. After one hour of incubation at room temperature, the antibody was drained off and the slides were rinsed once with PBS/BSA. Slides were washed three times over 45 minutes to 1 hour in PBS/BSA. For very abundant antigens, conventional fluorochrome-linked species-specific antibodies were used to detect the first antibody, at dilutions recommended by the manufacturer (Sigma or Zymed, South San Francisco, CA). $F_{(ab'2)}$ antibodies were used when possible to minimize background caused by F_c binding. For maximum signal from weaker antigens, including the integrins, the biotin-streptavidin system (Amersham International, UK) was used. Sections were incubated for 45 minutes in biotinylated or fluorochrome-conjugated second antibody at room temperature and washed as above.

If the second antibody was fluorochrome-linked, sections were mounted in DABCO (mounting solution; recipe below) after the final wash: 10-15µl

was put onto each section, a clean 13mm #1 coverslip placed slowly on top, to minimize bubbles, and coverslips were sealed with lurid-colored nail polish. If the second antibody was biotin-conjugated, sections were incubated in streptavidin conjugated to fluorescein isothiocyanate (FITC) or Texas Red for 15 minutes, washed and mounted as above. Slides were viewed immediately on a Zeiss Axiophot inverted stage UV photomicroscope with a filter at 450-490nm (for FITC) or 546nm band pass (for Texas Red/rhodamine), or stored at -20 °C. Staining was generally stable for at least 6 months when stored. Controls consisted of omitting the first antibody. Non-specific labeling was generally very faint, with the exceptions of the stratum corneum, which sometimes labeled strongly, and those noted in the results. Autofluorescence of the tissue was checked by viewing through the inappropriate filter for the fluorochrome used; autofluorescence was visible on both filters.

2.3.1 Dual labeling

To localize two antigens in the same section, sections were labeled with antibody against the first antigen, as detailed above, and then labeled with antibody — raised in a different species — against the second antigen, using a different fluorochrome (in both emission wavelength and species origin) from the one used to localize the first antibody. Where fixatives for the two different antigens were incompatible (i.e. co-localizing integrins and involucrin), sections were not fixed before labeling, and Triton X-100 was omitted from the PBS/BSA. These conditions were not optimal for either antigen, but still allowed discrimination of the staining patterns.

2.3.2 DABCO mounting solution

DABCO (1,4 diazabicyclo (2,2,2) octane) was added to a glycerol-based mounting medium to retard photobleaching. DABCO was added to 2.5% w/v in 90% glycerol, 50mM Tris pH 8.9, and 0.02% sodium azide (Howlett *et al*, 1987). Aliquots were stored at -20 °C.

2.3.3 Immunofluorescence photography

Staining was photographed on a Zeiss Axiophot photomicroscope, using Kodak Pan-X 125 ASA film (Kodak UK, Hemel Hempstead) with the camera set at ASA 400 and automatic exposure, and developed using Dia-Fine (New York, NY) developer and Ilford Hypam (Mobberley, UK) fixer. Kodak Ektachrome 400 was used for color photography. Printing of negatives, slide developing, and montage photography were performed by the ICRF Photography Unit.

2.4 Adhesion Assays

The adhesion of keratinocytes to different extracellular matrix (ECM) components was quantitated by allowing cells to attach to coated dishes for a defined period of time and counting the number of cells that adhered (Adams and Watt 1991). Cells used were subconfluent, since cells from confluent cultures are more difficult to trypsinize and have a reduced adhesion efficiency. Bacteriological (i.e. not treated for tissue culture) 96-well, square well plates (Linbro, Flow Laboratories, McLean, VA) were coated with purified ECM components at minimal (10μg/ml) or near-saturating (30μg/ml) concentrations (Adams and Watt 1991). Laminin was derived from the Engelbroth-Holm-Swarm murine sarcoma; collagen type IV was derived from human placenta; fibronectin (BPL, Elstree, UK) was derived from human plasma. All ECM components were stored according to manufacturer's instructions, and thawed as follows. Laminin was thawed at 4°C; collagen was thawed at room temperature; and fibronectin was thawed in a 37°C waterbath. ECM proteins were diluted in PBSABC so that the appropriate concentration could be added to each well in 100μl. Triplicate wells of each ECM for each cell type/condition to be tested were prepared, as well as triplicate control wells for non-specific adhesion prepared by "coating" wells with PBSABC only. Coating was allowed to take place overnight at 4°C, wrapped in Parafilm, or for one hour at 37°C.

Following coating, plates were washed three times in PBSABC. All wells were blocked with BSA (fraction V, Ig-free) as follows. A 10mg/ml solution in PBSABC was denatured at 80°C for 4 minutes, then diluted to 0.5mg/ml in PBSABC (Grinnell and Feld 1979). 100 μl was added to each

well and plates were incubated for 1-2 hours at 37°C. Cells were trypsinized and the 5ml trypsin/cell suspension added to 0.5 ml of 10mg/ml soybean trypsin inhibitor in serum-free FAD without HICE and counted. 1mg trypsin inhibitor inactivated 1.7mg trypsin; this corresponded to 8.5mg trypsin inhibitor per 1ml 0.25% trypsin. Trypsin inhibitor was used instead of serum to inactivate trypsin to avoid exposing the cells to serum fibronectin and vitronectin. Cells were spun down, resuspended in serum-free FAD and spun again. Pellets were resuspended at 10^5/ml for adhesion quantitated by visual counting, 2×10^5 for adhesion quantitated by hexosaminidase activity (see below), or 3.3×10^6 for adhesion quantitated by ^{51}Cr.

Plates were washed in PBSABC, and the last traces of PBS were removed by banging the inverted plate onto the bench. To quantitate adhesion by visual counting: for adhesion to fibronectin or collagen, 10^3 cells in 100 µl were added per well (i.e. cells were resuspended at 10^4/ml); since adhesion to laminin or BSA was lower, 10^4 cells in 100 µl were added per well (ie cells were resuspended at 10^5/ml). To quantitate adhesion by hexosaminidase activity, 2×10^4 cells in 100 µl were added per well (i.e. cells were resuspended at 2×10^5/ml) for all ECM types, as it was not necessary to have densities that were reasonable to count by eye. ^{51}Cr adhesion assays are discussed below. Plates were placed in a 37°C incubator for 3 hours.

For plates to be quantitated by hexosaminidase activity, plates were washed gently three times in PBSABC and hexosaminidase substrate was added (method follows). For plates to be quantitated by visual counting, medium containing non-adherent cells was flicked out, and 100 µl of freshly diluted 4% formaldehyde in PBSABC was added to each well and plates were left on a gently shaking platform for 45 minutes. Plates were washed three times in PBSABC, and stained with filtered 1% methylene blue in PBS for 45-60 minutes at room temperature. Plates were then washed twice with distilled water and allowed to dry.

Cells in each well were counted (three fields per well on 10X objective, or five fields on 20X for high cell densities) using a graticule with a square to count the same area for each well. Results were expressed as a percentage of the cell number originally added. To extrapolate results to whole wells,

counts were corrected using the following multiplication factors, where the area of well= 28.27mm^2:

	10X objective:	26.9366
	20X	109.4464

2.4.1 Hexosaminidase assay for cell number

To quantitate the number of adherent cells, this method has the advantage over direct counting of greater ease and consistency. It depends on the conversion of a substrate by a cytoplasmic enzyme to a color product dependent only on cell number (Leitriz *et al*, 1992) and can be accurate to a few hundred cells (Givens *et al*, 1990). Cells were prepared as described above. For quantitation of adherent cells, and as a control for cytokine treatment effects on hexosaminidase activity, a standard curve was constructed: cells in suspension were aliquoted to known numbers (range of 10^3-10^5 cells in linear steps), in a final volume of up to 1.5ml serum-free FAD. PBSABC was added to make the final volume 1.5ml and cells were spun down; the pellets were kept at 37°C until the three hour adhesion period was finished. The pellets were then resuspended in 60µl NPAG solution (below) and incubated as for adherent cells.

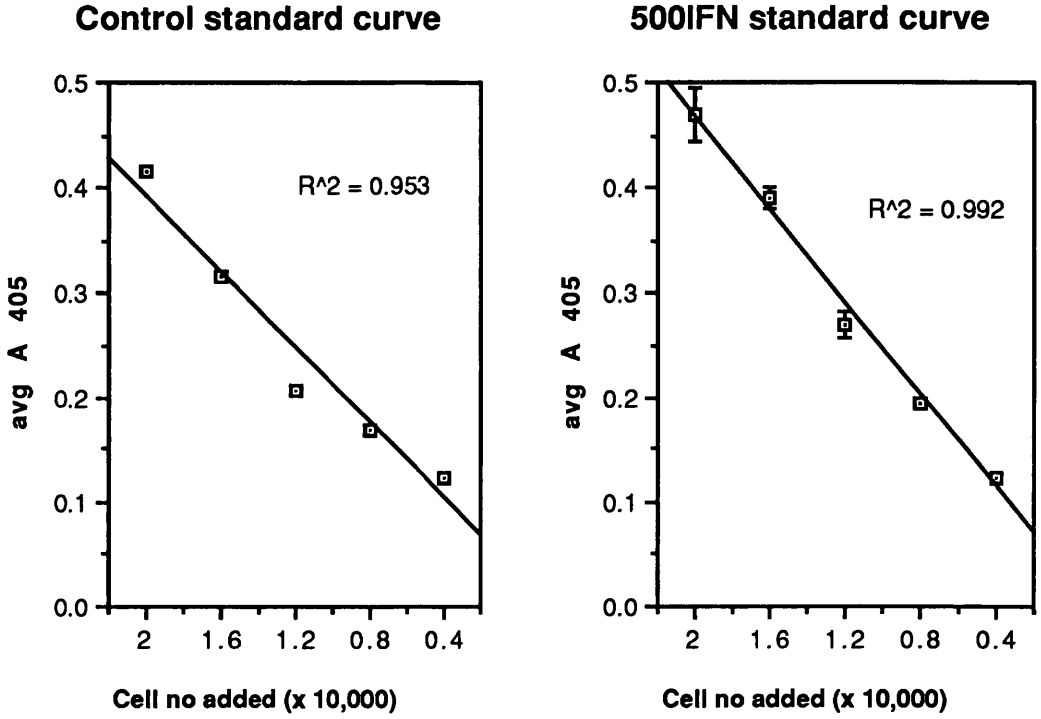
Following the three hour adhesion, wells (with the adherent cells) were washed three times in PBS, and 60µl of NPAG solution was added; plates and standards were incubated for 4-16 hours, at 37°C in a humid chamber. Alternately, the plates could be flicked dry and stored at -20 until the assay could be performed, ideally the next day. The cell standards were refrigerated, not frozen if stored. Representative standard curves are illustrated below.

To stop the reaction and develop the color, 90 µl of 50mM glycine, pH 10.4 and 5mM EDTA solution was added to plates and standards. The standards were transferred to 96-well plates and all samples were read at 405nm in a plate reader (Titertek Multiskan MCC/340 II, Flow Laboratories).

NPAG solution

A 7.5mM solution of NPAG (p-nitrophenyl N-acetyl-β-D-glucosaminide) in 0.1M sodium citrate, pH 8.0 (titrated with citric acid) was added to an

equal volume of 0.5% Triton X-100 in distilled water, aliquoted, and stored at -20°C.

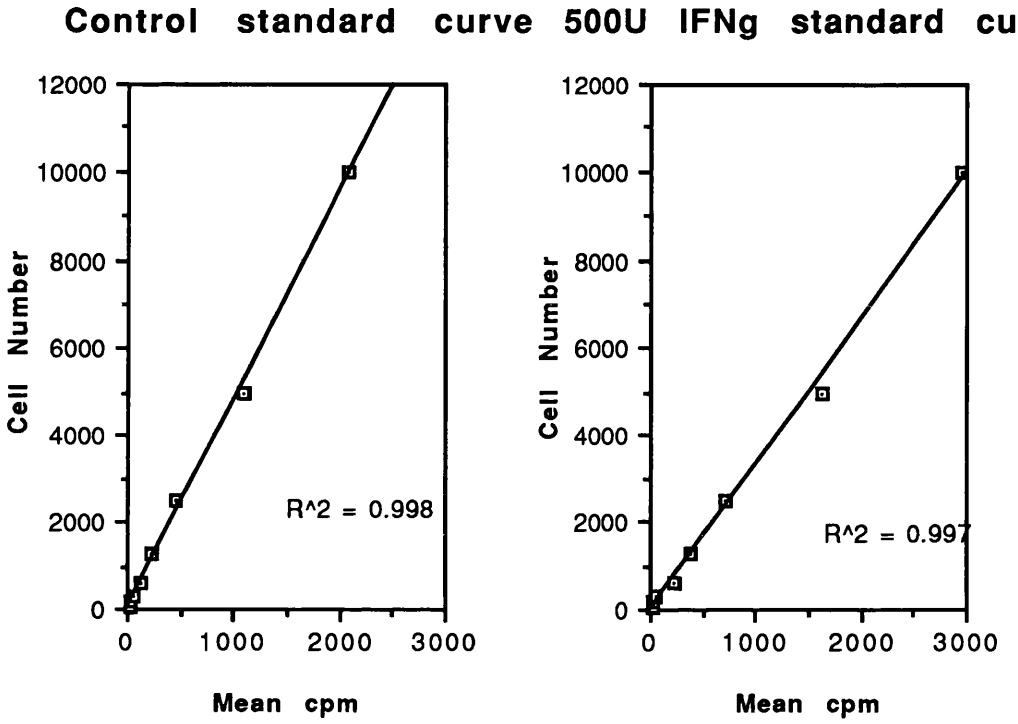


2.4.2 ^{51}Cr assay

Cells passively take up radioactively labeled chromate, dependent on size and permeability, and cell number can therefore be measured by quantitating internalized ^{51}Cr (Brunner *et al*, 1976). Cells were trypsinized, counted and washed as above, then 10^6 cells were resuspended in 300µl serum-free FAD, and 100µCi of aqueous sodium chromate (Amersham # CJS1) was added. Cells were labeled for 45 minutes at 37°C, tapping every 15 minutes to resuspend cells. Cells were then washed twice in serum-free FAD to remove unbound ^{51}Cr. The pellet was resuspended at 2 x 10^5/ml , and 50 µl (i.e. 10^4 cells) was added to ECM-coated wells, already containing 50µl serum-free FAD and prepared as described above. Because of the increased sensitivity of this assay, one hour at 37°C was sufficient for quantitation (Neil Hotchin, pers. comm.)

Cell standards, where a known number of cells was added to a counting vial, were prepared at a doubling dilution from 10^4 down to 78 cells, to

allow quantitation of the number of cells adhered. Following the one hour adhesion, plates were washed 3 times in PBSABC, and 75µl lysis buffer (0.1M NaOH, 1% SDS, and 2% Na_2CO_3) was added. Lysates were soaked up with a cotton bud and counted in a gamma counter (Pharmacia LKB 1261 Multigamma). A standard curve was constructed (examples below) and from this the number of adherent cells could be determined.

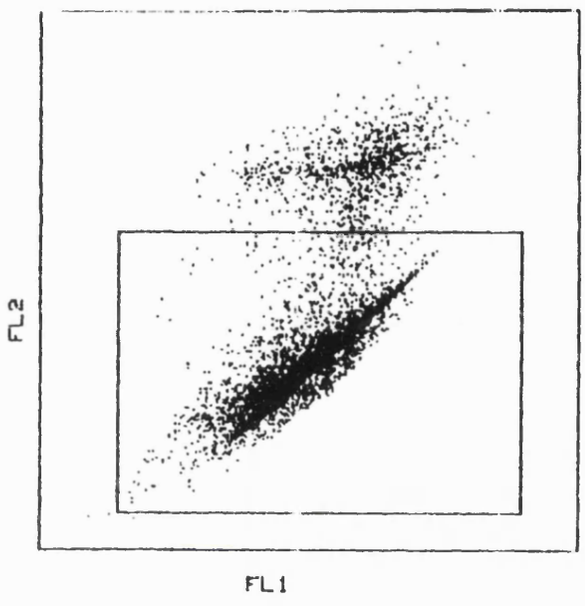


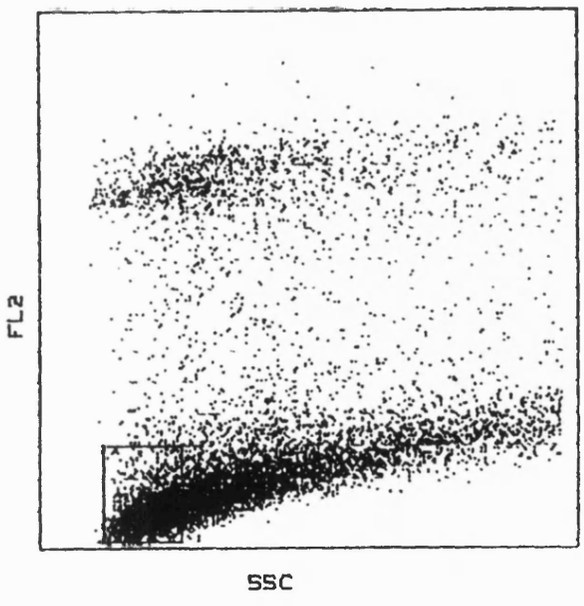
2.5 Keratinocyte staining for flow cytometry

This method was as developed by Philip Jones, Keratinocyte Lab. Keratinocytes to be stained were subconfluent, partly to aid in geting a good single cell suspension, but also because adhesion ability and integrin levels change on confluence (P. Jones, unpub. obs.). Cultures were treated as described in Chapter 5. Feeders were removed with versene and keratinocytes were trypsinized, making especially certain to get a single cell suspension. Cells were spun down in serum-containing FAD, and pellets were washed in 15ml ice-cold PBSABC. Pellets were resuspended in 1.25ml cold PBSABC and aliquotted at 150µl in 12 x 50mm polystyrene tubes (Falcon 2054) for staining. A minimum of 2×10^5 cells was stained. The sample was resuspended in ice-cold PBSABC so that the appropriate cell number was in a volume of approximately 150µl. All samples and

Figure 1 Representative gates used during flow cytometry data analysis. To exclude dead cells, as judged by propidium iodide uptake, a gate (box in upper panel) was set to exclude cells with high PI uptake (on the Y axis; FL2). FL1, on the X axis, is intensity of staining with the anti-integrin antibody used (anti-α_5 in this case).

To exclude both dead and suprabasal cells, the lower gate (small box in lower left-hand corner) was applied. PI staining is again FL2, on the Y axis. Cell "density" (called side scatter or SSC; X axis) increases with differentiation; gating on low SSC values results in selection of >80% basal cells (P. Jones, unpub. obs.)


FL2
FL1


FL2
SSC

solutions were now kept ice-cold to minimize antibody internalization and cell death. Originally, cell samples were filtered through 100μm nylon mesh before staining to eliminate clumps and debris. This was discontinued, however, as it proved unnecessary as unfiltered samples did not block the cytometer, and the removal of large, differentiated cells was avoided by not filtering. Each sample was added to antibody (in a volume of 20 μl PBS) already placed in the tube. When possible, directly labeled antibodies were used, as the weaker signal could be amplified by the cytometer, and time and therefore loss of viability was reduced. Directly-labeled antibodies (conjugated by P. Jones) used were: mouse monoclonal anti-CD29 (Janssen, UK) against the β_1 subunit; mouse monoclonal HAS-4 against α_2; and mouse monoclonal VM-2 against α_3 (see Table 1 for sources). Other, indirectly labeled antibodies used were: rat monoclonal mAb 16 against α_5 and rat monoclonal GoH3 against α_6 (see Table 1 for sources). Antibodies were used at the same dilutions as for immunofluorescence. For each sample, a cell-surface antigen control for non-specific binding was also prepared. FITC directly labeled monoclonal anti-CD8 (a T-cell receptor for MHC class I (Bierer and Burakoff 1988); Sigma) was used for mouse monoclonal surface marker antibodies; goat-anti rat FITC labeled antibody alone was used for rat monoclonal surface marker antibodies. Cells were incubated with antibody for 20-30 minutes, then washed by adding 4ml PBSABC to each tube, inverting, and centrifuging 5′ at 1000 rpm at 4°C. For directly labeled antibodies samples were resuspended an appropriate volume for analysis (~200μl). Samples were then ready to run. For indirect antibody labelling , the cells were resuspended in 150μl of FITC-conjugated antibody, added at the appropriate dilution (1/100). Cells were incubate 10-20 minutes, then washed and resuspended as above.

Labelled cells were analyzed in a Facscan flow cytometer (Becton-Dickinson, San Jose, CA), acquiring 10,000 events with settings previously determined for keratinocytes. Typical settings were as follows, where FSC is forward scatter (related to cell size), SSC is side scatter (related to cytoplasmic complexity), FL1 is fluorescence detector 1 (set for FITC, i.e. anti-integrin fluorescence), and FL2 is fluorescence detector 2 (set for propidium iodide, taken up by permeable cell DNA). FSC and SSC were collected with linear amplification, FL1 and FL2 with logarithmic amplification. Cytometer detector settings were: FSC E00; SSC 291; FL1

386; and FL2 349. Amplification was set at 4.79 for FSC; other parameters were unamplified. The forward scatter threshold, below which data were ignored to exclude small debris, was set at 52. Immediately before running samples, propidium iodide (PI) was added to a final concentration of 50μg/ml, to stain dead cells. Debris and large cell aggregrates were live gated, so that only single or double cells were acquired. Typical dot plot scattergrams for untreated, normal keratinocytes are illustrated in Fig. 1.

Analysis was performed by gating out dead cells: plotting FL1 vs. FL2 (see Fig. 1, upper gate), yielding statistics for all live cells, and also by gating out dead and differentiated cells, by plotting FL2 vs. side scatter, yielding statistics for basal, live cells (Fig. 1, lower gate). Differentiated keratinocytes have a higher side scatter value; their elimination allows enrichment for basal cells. Treated keratinocytes were compared to normal keratinocytes by normalizing modal values and the results expressed as percentage change from control levels.

2.6 Protein chemistry

2.6.1 Metabolic labeling of keratinocytes

To label newly synthesized proteins for detection and quantitation, cultured keratinocytes were labeled overnight with ^{35}S-methionine and cysteine (TranS^{35}-Label, ICN, specific activity 1180Ci/mmol) in FAD + FCS +HICE, at 50 μCi/ml (Hotchin and Watt 1992). Shorter labelling times, e.g. for pulse-chases, were carried out in methionine and cysteine-free medium. Methionine and cysteine-free FAD medium was not available; DME was used for the short labeling period, supplemented with HICE, 6% FCS that had been dialyzed against PBS, and 4% normal FCS.

^{35}S-methionine-cysteine label ("label") was thawed; at the same time, if keratinocytes were to be pulsed, they were depleted of endogenous methionine and cysteine by incubating in met-cys free medium for 1 hour. Keratinocytes were labeled for the desired time (2 hours for pulse chase, or overnight) in 50μCi/ml. Flasks were sealed and placed into a sandwich box with a 60mm dish of activated charcoal (to absorb volatile radioisotope) and incubated at 37°C.

After labelling, flasks were rinsed in normal FAD + FCS + HICE, then incubated further in FAD + FCS + HICE with 5mM cysteine and methionine added if indicated, to "chase" the label (allow it to be processed) into mature protein. A 5-12 hour chase will allow precursor and mature labeled protein to be isolated from the same cells. Cells were then harvested, first removing the feeders with versene. An aliquot of cells was sometimes removed for use in an adhesion assay. To lyse the cells, all medium was removed and 100µl lysis buffer/10^6 cells was added, and the lysate was scraped off with a disposable cell scraper. Lysates were transferred to pre-cooled microfuge tubes and incubated on ice for 20 minutes. In all subsequent steps, samples were kept on ice as much as possible; proteases are highly temperature dependent and will be inhibited by cold. Lysates were spun 1-2 minutes in a refrigerated microfuge at 13,000 rpm to remove cellular debris. The supernatant was divided into 100µl aliquots in pre-cooled microfuge tubes and stored at -70°C.

NP-40 lysis buffer consisted of 150mM sodium chloride, 1.0 % NP-40, and 50mM Tris pH 8.0 (Harlow and Lane 1988; Adams and Watt 1991; Hotchin and Watt 1992). Just before use, protease inhibitors were added: leupeptin was used at 1 µg/ml and phenylmethylsulfonylfluoride at 2mM.

2.6.2 Immunoprecipitation of integrins

One 100µl aliquot of each sample was thawed and put on ice. Duplicate 5µl aliquots of each sample were put into 5ml cold 10% trichloracetic acid (TCA) and incubated for 10 minutes. Precipitates were filtered through 10% TCA-wetted glass fiber filters on a Millipore multiwell apparatus; unprecipitated material was washed from the filters with two 5ml cold 2% TCA washes. Filters were dried at 37°C, then immersed in 5ml scintillant (Ecolume, ICN) and counted on a Beckman Model LS1801 liquid scintillation counter. In addition, total protein content was determined using the Bradford assay (see below).

The volume per sample of lysate equal to 10^6 cpm or 40-60µg protein was brought to a 100µl final volume with lysis buffer containing protease inhibitors. Initially, a duplicate of each, for a control immuno-

precipitation with irrelevant antibody of same isotype (anti-CD3, the T-cell receptor, not expressed by keratinocytes, IgG_{2a} isotype, Sigma), was also included. 0.4µg of CD29 or CD3 antibody, or 5µl of anti-ICAM-1 was added to each sample and incubated at least 90 minutes on ice.

40µl hydrated 50% protein A sepharose (PAS; Pharmacia) was added to each sample and incubated at least 60 minutes on a rotating wheel in the cold room. For IgG_1 antibodies, i.e. anti ICAM-1, Protein G sepharose (Pharmacia) treated as per PAS, was used. To swell PAS, approximately 50µl of dry material was hydrated with 1 ml sterile, distilled water and incubated for 20-30 minutes at room temperature. Hydrated PAS was spun down at 13000 rpm, resuspended in sodium phosphate (PO_4-) buffer (below), and washed twice more. PAS was resuspended in a volume approximately equal to the packed pellet and stored at 4°C until use.

Samples were spun for 30 seconds at 13000 rpm and the supernatant was aspirated. Pellets were washed (Adams and Watt 1988) in cold solutions as follows: once with 1ml PBSABC + 0.5% TX-100 + 0.1% SDS; once with 1ml PBSABC + 0.5M NaCl; and twice with 1ml PBSABC + 0.5% TX-100 + 0.1% SDS, aspirating as much of the supernatant as possible.

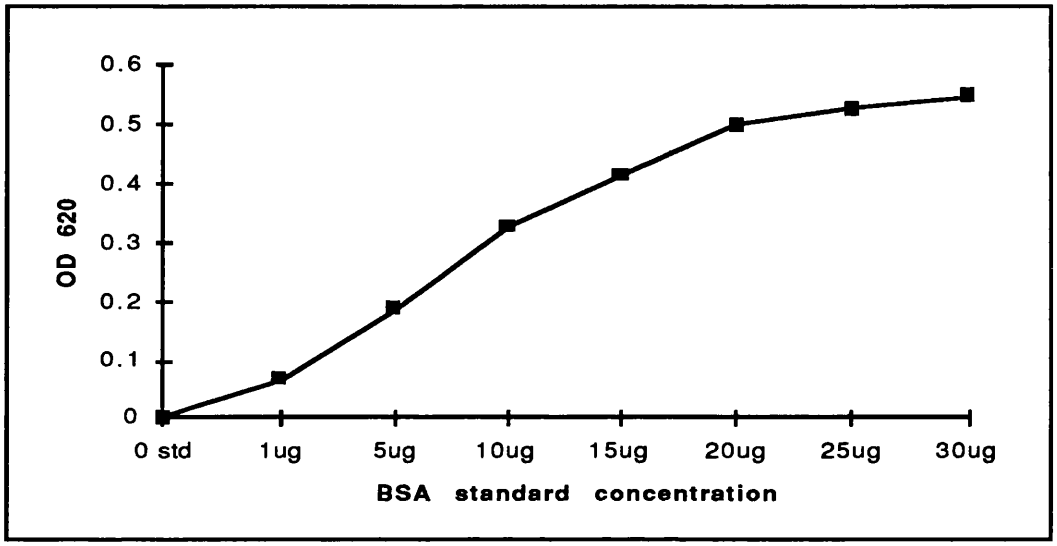
50µl of sample buffer was added to each pellet and mixed. Samples were frozen at -70°C, or boiled 3-5 minutes to denature the proteins just before loading on the gel. Frozen samples were thawed and then boiled just before loading. High molecular weight pre-stained markers (Bio-Rad) were treated the same way. Samples were spun down and the supernatant (55µl) was loaded onto a 7.5% SDS polyacrylamide gel (below).

Sample buffer consisted of 2.0ml 10% SDS, 2.0ml glycerol, 100µl 0.4% bromophenol blue, and 3.2ml distilled water (Harlow and Lane 1988). Aliquots were stored at -20°C.

0.1M PO_4 buffer, pH 8.0 consisted of 47.35ml 0.2M NaH_2PO_4, 2.65ml 0.2M Na_2HPO_4, and 50.0ml H_20 (Sambrook *et al*, 1989).

2.6.3 Bradford assay for protein content

This assay uses the color conversion of a substrate dependent on protein concentration to quantitate protein content (Bradford 1976). Bradford reagent (Bio-Rad, Richmond, CA) was diluted 1:5 just before use. A standard curve was constructed using bovine serum albumin (BSA; fraction V) concentrations of 0, 1, 5, 10, 15, 20, and 30 µg. If the unknown



protein concentration sample was extracted in lysis buffer (e.g. metabolically labelled lysates), a volume of lysis buffer equal to the volume used in the Bradford assay (usually 5 µl) was added to each standard. BSA standard or unknown sample was added to 1ml diluted Bradford reagent, inverted several times, and quadruplicate wells of 200µl each prepared in a square-bottom 96-well plate. The unknown was assayed in duplicate. Optical density at 620nm was read in a Titertek plate reader and unknown protein concentration determined against the standard curve (a typical standard curve is illustrated above). If the volume of unknown used contained a protein concentration outside the linear range of the standard curve, more or less as appropriate was used and the assay repeated.

2.6.4 Polyacrylamide gel electrophoresis of proteins

1.5 mm thick polyacrylamide gels were run on a vertical electrophoresis gel apparatus (Model SE 400, Hoefer Scientific Inc., San Francisco, CA),

using detergent and methanol/acetone-cleaned glass plates, assembled according to the manufacturer's instructions. The resolving gel solution was prepared as described below (method based on (Laemmli 1970)) and 25ml was poured at least one hour before running. The gel surface was immediately overlaid with 1-2 ml 0.1% SDS, to eliminate an air interface which prevents acrylamide polymerization. Gels were allowed to polymerize at least 45 minutes.

Before the samples were to be run, the SDS was poured off and a 5% acrylamide stacking gel solution (below) was added. A 15-well comb was inserted and the solution was allowed to polymerize at least 15 minutes. Buffer (below) was added to within about 3cm from the top, and any air bubbles trapped under the gel sandwich were flushed out with a pipette. The wells were rinsed with running buffer and filled. Samples were boiled and immediately added to the wells, using capillary tip disposable pipette tips. Running buffer was gently added to the top tank and the gel was run at 40-50V constant voltage and approximately 10mA overnight. Running was stopped when the dye front was about 1 cm from the end.

The gel was carefully removed from the plates and stained 30 minutes in 0.1% Coomassie Blue, made in 10% acetic acid and 50% methanol (Harlow and Lane 1988). Gels were destained 2 hours, with at least one change, in 10% acetic acid and 5% methanol until the markers were visible. The gel was then placed in Amplify (Amersham International, UK), to enhance fluorography, for 30 minutes, then dried down for 2 hours at 80°C in a vacuum gel drier (Bio-Rad Model 543) onto 3MM chromatography paper (Millipore). Dried gels were placed in an X-ray cassette and taped down. A phosphorescent marker strip (Stratagene, Cambridge, UK), flashed twice with a flash unit, was placed next to the gel for orientation and identification of the markers. XAR-5 X-ray film (Kodak) was placed on top and exposed at -70°C for 2-3 days, with longer exposure if necessary with a fresh piece of film.

Resolving gel solution (7.5%) consisted of 14.4 ml sterile distilled water, 7.5 ml 1.5M Tris-OH pH 8.8, 7.5 ml 30% acrylamide/bisacrylamide, 300 µl 10% SDS, 300 µl 10% ammonium persulfate, and 30 µl TEMED.

Stacking gel (5%) consisted of 5.8 ml sterile distilled water, 2.5ml 0.5M Tris pH 6.8, 1.5ml 30% acrylamide/bisacrylamide, 100µl 10% SDS, 100µl 10% ammonium persulfate, and 10µl TEMED.

Running buffer consisted of 6g Tris-OH, 28.8g glycine, brought to 990ml in distilled water, and 10ml 10% SDS.

2.7 Molecular biology

2.7.1 Buffers and solutions used

2.7.1.1 20X sodium saline phosphate EDTA (SSPE)

Prepared as described in (Sambrook *et al*, 1989), SSPE consisted of 3.6M NaCl, 0.02 M EDTA pH7.7, and 0.2 M NaH_2PO_4 prepared in distilled water and autoclaved. For use with RNA, solution was treated with DEPC as described.

2.7.1.2 20X sodium saline citrate (SSC)

20X SSC (ICRF) consisted of 3M NaCl and 0.3M trisodium citrate, which was autoclaved.

2.7.1.3 Phenol equilibration

Molecular biology grade, redistilled phenol (Gibco-BRL, Paisley, Scotland) was stored at -20° C until needed. Phenol was thawed and heated to 68° C, and 8-hydroxyquinoline was added to 0.1%, to retard oxidation (Sambrook *et al*, 1989). An equal volume of 0.5M Tris, pH 8.0 was added and the bottle was shaken vigorously. The Tris was aspirated and replaced twice, or until the Tris pH was 7.8 or greater and then replaced with 100mM Tris, pH 8.0. Equilibrated phenol was stored at 4° C up to three months.

2.7.1.4 Tris-borate electrophoresis buffer (TBE)

TBE, used as the buffer for urea polyacrylamide RNA gels, was made as a 20X stock and autoclaved. 20X TBE consisted of 216g Tris base, 110g boric

acid, and 80ml 0.5M ethylenediaminetetraacetic acid (EDTA) (Sambrook *et al*, 1989).

2.7.1.5 Tris-acetate electrophoresis buffer (TAE)

TAE, used as a buffer for agarose gels, was made as a 50X stock and autoclaved. 50X TAE consisted of 242g Tris base, 57.1ml glacial acetic acid, and 100ml 0.5M EDTA pH 8 per liter (Sambrook *et al*, 1989).

2.7.1.6 SOC medium

SOC medium, for bacterial transformation, was prepared as follows: per 100ml, 4.0g Bacto-tryptone (DIFCO Laboratories, Detroit, MI), 1.0g yeast extract (DIFCO), and 0.1g NaCl were added and the final volume brought to 100ml, then autoclaved. Then 1ml of $MgCl_2/MgSO_4$ solution was added. $MgCl_2/MgSO_4$ solution consisted of 12.0g $MgSO_4 \cdot 7H_20$ and 9.5g $MgCl_2$ brought to 100ml with distilled water and filter sterilized. Just before use, 1ml of 2M sterile glucose was added and the solution filter-sterilized.

2.7.1.7 100X Denhardt's Reagent

100X Denhardt's consisted of 2% w/v bovine serum albumin, 2% w/v Ficoll (MW 400,000), and 2% w/v polyvinylpyrollidone (Sambrook *et al*, 1989). This was aliquoted and stored at -20° C.

2.7.1.8 Tris-EDTA (TE)

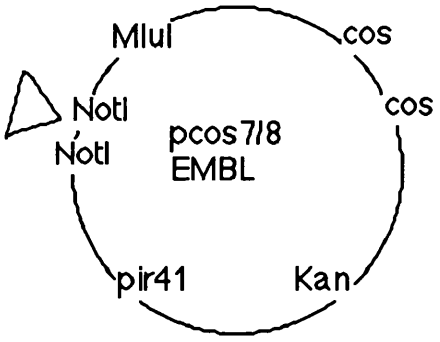
The pH of TE was determined by the pH of the Tris buffer used. TE consisted of 10mM Tris-Cl at the appropriate pH and 1mM EDTA, pH 8.0 (Sambrook *et al*, 1989).

2.7.2 DNA transfection

Two methods were used for transfection of construct DNA: ES cells were electroporated, and STO cells were lipofected. Lipofection did not require

development of optimal transfection conditions and STO cells were expected to be readily transfectable by this method.

2.7.2.1 DNA used for transfection



Plasmid pSV2neo, as originally described by (Southern and Berg 1982), was obtained from Dr. X.-F. Pei, ICRF. This is a pBR322 derivative, encoding the ampicillin resistance gene and the *neo* gene, driven by the SV40 origin. The cosmid pcos7/8 EMBL is unpublished, but was originated by Dr. Alaister Craig, University of Oxford. It was obtained as the vector for the human genomic library prepared by Dr. Anna-Maria Frischauf, ICRF, who generously donated it. A simplified map of pcos7/8 EMBL is illustrated above. The human genomic fragments (represented by a triangle) were cloned into the *Not* I sites; the cosmid was amplified by growing the host on kanamycin agarose-LB plates. Kanamycin was used for bacterial selection, but G418 was used for selection of ES transfectants, as the *kan* gene product also confers resistance to G418. Four fractions, A, E, F, and G were provided. Fraction A contained 10^5 independent recombinants, E contained 8×10^4, F contained 2×10^5, and G contained 1.6×10^5. Complexity was checked by cutting with *Mlu* I followed by agarose gel analysis. High molecular weight smears confirmed a range of DNA fragments (not shown). For transfection, cosmid DNA was cut with *Mlu* I, to linearize the vector. *Mlu* I was chosen as it was least likely to cut within the genomic fragments. The pcos8 cosmid vector alone was also obtained and grown up for use in transfections.

2.7.2.2 Electroporation of ES cells

Electroporation has been described as the most efficient way to introduce foreign DNA into ES cells (Doetschmann *et al*, 1988; Wagner 1990). This

protocol was optimized for the CCE line of ES cells. DNA to be introduced was as uncontaminated as possible, and linearized. Cells to be electroporated were low passage, if possible, and sub-confluent i.e. day 2 of ES culture. The cells were fed 2 hours before trypsinization. Cells were then washed once in PBS (room temperature), pelleted, and resuspended at 4×10^7 cells/ml in PBS. 500µl of cell suspension was added to an electroporation cuvette (Bio-Rad; 0.4cm electrode gap). 25µg of DNA was added and the contents of the cuvette were well mixed. Cuvettes were electroporated at 200 volts and 960 µF capacitance (Andreason and Evans 1988; Doetschmann *et al*, 1988; Schwartzberg *et al*, 1989) corresponding to 625V/cm. Samples were held at room temperature for 10 minutes following electroporation. Electroporated cells were plated at 5×10^6 cells per T25 on G418-resistant STO feeders and fed 1 and 2 days after electroporation. Cells were then either passaged into 200µg/ml G418 (Gibco-BRL; actual drug concentration of about 100µg/ml) or the medium was changed to G418-containing medium. Determination of the optimal concentration of G418 is described in Appendix 2. Passaging was best, as the cells cross-fed well as colonies and non-transfected cells took longer to die. Selection was continued in G418 for at least 2 passages and then at alternate passages.

2.7.2.3 Lipofection

Lipofection, which involves transmembrane transport of DNA in lipid vesicles (Felgner *et al*, 1987), was performed as described by the manufacturer (Gibco). DNA was diluted to 1, 5, or 10µg in 50µl of sterile distilled water, and Lipofectin was diluted to 40µg in 50µl of sterile distilled water. These solutions were combined in a Falcon 2003 polystyrene tube, mixed gently, and allowed to stand at room temperature for 15 minutes. STO cells, 50% confluent in 60mm dishes, were washed twice with serum-free DME, and 3.0ml of DME was added to the cells. The Lipofectin-DNA complex was added to the dish, dropwise, as the plates were swirled. The cells were incubated for 20 hours, then the medium was changed to DME + 20% DCS. Cells were incubated for 5 days, until confluence was reached, before selection with G418 at 500µg/ml was started. Determination of the optimal concentration of G418 is described in Appendix 2.

2.7.3 *In Situ* RNA Hybridization

This method was developed for ^{35}S-labeled probes against human integrin subunit mRNAs and is based on the method by Craig Thompson, NIH; and (Angerer *et al*, 1987; Naylor and Balkwill 1992)); and on discussions with Richard Poulsom, ICRF. All plasticware used had been γ-irradiated and solutions were treated with 0.1% diethylpyrocarbonate (DEPC) as described in (Sambrook *et al*, 1989).

2.7.3.1 Probe subcloning

2.7.3.1.1 Integrin probes

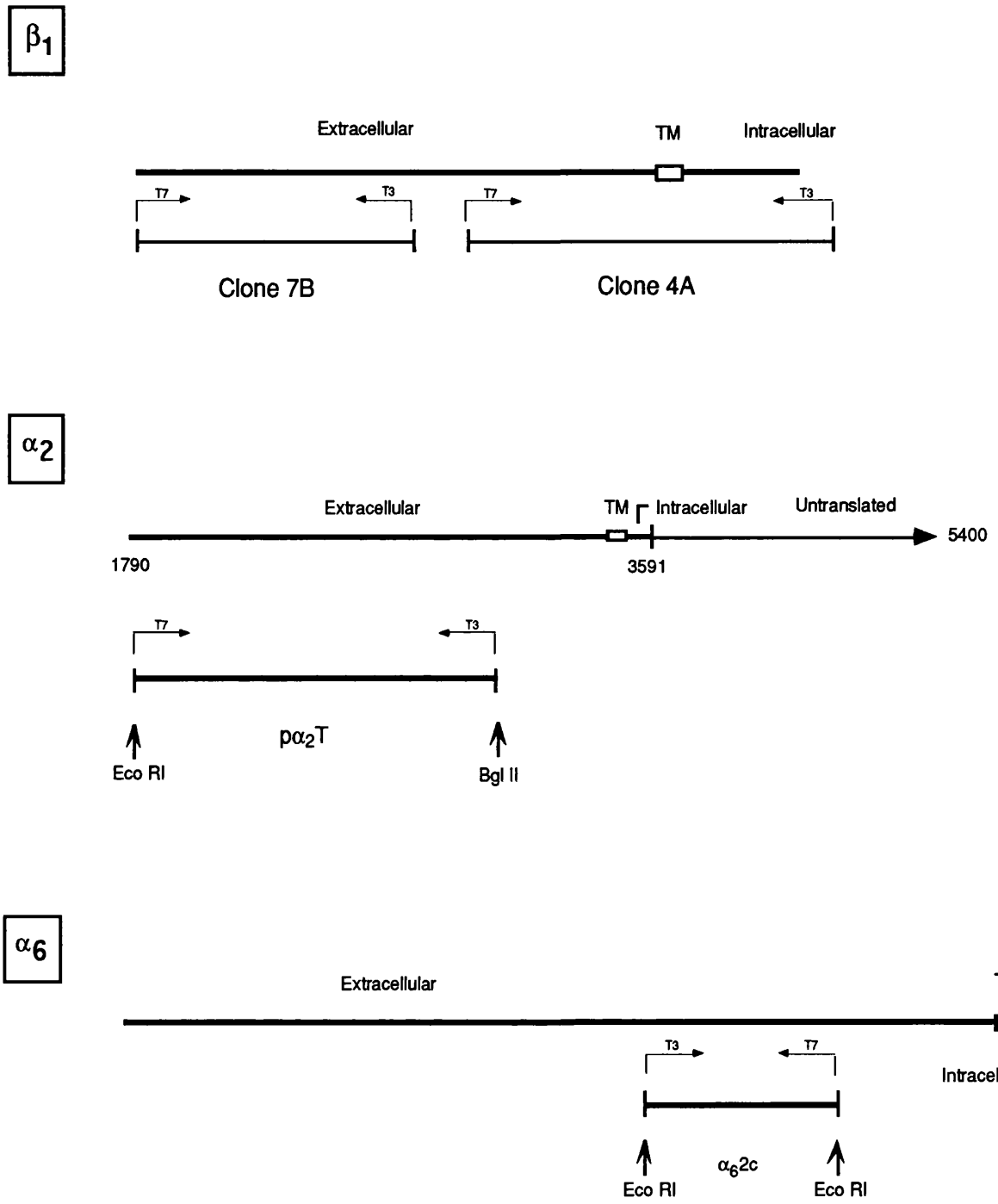
Most of the cDNAs I wanted to use as probes needed to be subcloned, either to place the cDNA into a vector which supported transcription of the cDNA, or to place a shorter cDNA (*in situ* probes are ideally less than 1 kilobase (Kb) (Angerer *et al*, 1987)) into a transcription vector. In all cases, I used the Bluescript II vector (Stratagene), as it was the most versatile vector. When the subcloned insert is cut out as a cassette, using *Bss* HII, the RNA polymerase sites are retained at their respective ends; thus, transcribing with one of the enzymes results in transcripts in one direction only, with the addition of 172 base pairs (bp), consisting of the multicloning and polymerase sites. Standard methods as described in (Sambrook *et al*, 1989) were used.

2.7.3.1.2 β_1

The cDNA probes (pMINTβ; (Holers *et al*, 1989)) used for subcloning were obtained from Dr. Richard Hynes, MIT, Boston, MA (Clone 7B, 1 Kb) and from Dr. Douglas DeSimone, Univ. of Virginia, Charlottesville, VA (Clone 4A, 1.3 Kb), and are derived from the mouse integrin β_1 subunit (see Fig. 2). Cross-species β_1 homology is very high (DeSimone and Hynes 1988), and the cDNA probe had been used in the lab to probe human RNA in Northern blots (e.g. (Nicholson and Watt 1991)).

Figure 2 Constructs used as *in situ* probes. β_1: the full length cDNA map as provided by Dr. D. DeSimone; cDNA subclones indicated below. α_2: partial cDNA map of clone p$\alpha 2_{2.72L}$; representing the 3′ end of the cDNA and a large 3′ untranslated region; with *in situ* subclone (pα_2T) below. α_6: full cDNA map of the A form of α_6 (Hogervorst *et al,* 1991); with *in situ* subclone ($\alpha_6$2c) below. **β-actin**: partial cDNA map of clone pHFβA-1; representing small 5′ untranslated portion, with transcription start indicated. This clone represents just the 5′ portion of the gene.

Extracellular = extracellular domain of translated protein
TM = predicted transmembrane domain
Intracellular = intracellular domain of translated protein
RNA polymerase (T3 or T7) used to generate sense (5′→ 3′ transcription) or anti-sense (3′→ 5′ transcription) is indicated above subclone map.


β1
Extracellular
TM
Intracellular
T7
T3
T7
T3
Clone 7B
Clone 4A
α2
Extracellular
TM
Intracellular
Untranslated
5400
1790
3591
T7
T3
pα2T
Eco RI
Bgl II
α6
Extracellular
TM
T3
T7
Intracellular
α62c
Eco RI
Eco RI
β-actin
Pst I
Ava II
transcr.
start
gene continues, but not cloned
β-act
200 bp

2.7.3.1.2.1 Clone 7B

This clone, covering most of the extracellular domain of the mouse β_1 integrin (see Fig. 2), is named pMINTβ_1 by its originators, and more specifically Clone 7B (EMBL/Genbank accession #Y00818). Since the original construct used pGEM1 (Promega, Madison, WI) as the vector, RNA transcription was possible. Cutting the construct with *Eco* RI allowed transcription with SP6 RNA polymerase (yielding anti-sense transcripts), and cutting with *Bam* HI allowed transcription with T7 RNA polymerase (yielding sense transcripts). SP6 transcription *in vitro*, however, resulted in very short transcripts (as judged by very little TCA-precipitable material, ie. synthesized polynucleotide), even though identical reaction conditions using T7 were able to transcribe the insert, and SP6 could transcribe the vector without an insert. Cutting the construct for SP6 transcription with another enzyme, *Bal* I, which cuts 59bp from the T7 initiation site, also did not result in acceptable transcription. After discussion with Dr. DeSimone, the most likely reason seemed that the insert cDNA contained a stop codon in the anti-sense direction, and it would be better to try using another cDNA (clone 4A, described below), which he provided.

While I was working on Clone 4A (below), I subcloned the entire Clone 7B insert into Bluescript II, in an attempt to improve the transcription efficiency. Since the insert ends were compatible with insertion in either orientation, it was necessary to determine the orientiation in order to choose the appropriate enzyme for anti-sense transcription. Cutting with an enzyme which cuts both within the insert and the vector would give a unique pattern of fragment lengths, dependent on the insert orientiation. Cutting complete plasmid with *Hind* III gave fragments that indicated T3 transcription would be in the anti-sense direction.

With this construct, transcription off both promoters proceeded efficiently, in contrast to the insert in the original.

2.7.3.1.2.2 Clone 4A

This clone covers the cytoplasmic and transmembrane domains of β_1 (see Fig. 2). The insert, at 1300 bp, was large for efficient full-length transcription, but I did not consider subcloning it essential since it was already in a transcribable vector (pGEM1). Digestion with *Bam* HI, generating a T7 transcribable fragment, was predicted to give the sense transcript, while digestion with *Pvu* II, yielding a T3 transcribable fragment, was predicted to give the anti-sense transcript.

2.7.3.1.3 α_6

This construct, obtained from Dr. Arnoud Sonnenberg (an *Eco* RI-*Eco* RI fragment from clone A33 (Hogervorst *et al*, 1991)), was an appropriate length for *in vitro* transcription (682 bp), but was inserted into a non-transcribable vector. I subcloned the entire insert into Bluescript II, cutting the original cDNA out of pUC18 using *Eco* RI. This generated a 682bp fragment which I subcloned, ultimately resulting in a construct I named $\alpha_6$2c (Fig. 2). To determine the direction of insertion, I cut $\alpha_6$2c with *Fok* I, which would give unique fragments of 1923 and 211 bp if inserted in the 5′→ 3′ direction. This is what I obtained, indicating that transcription with T7 would give the anti-sense.

2.7.3.1.4 α_2

This construct, originating from Dr. Hemler's lab (pα_2 2.72L; Accession #X17033; (Takada and Hemler 1989)) covering bases 1790 through 3590 plus a 3′ untranslated region of ~ 1780 bp was also too large, at 3.6Kb, for efficient full-length transcription. I cut the original insert with *Eco* RV, generating a 1327 bp fragment representing the 5′, translated region corresponding to 1790-3110 of the original clone. I then cut with *Bgl* II-*Eco* RI, finally yielding a fragment of 1083 bp from the 5′ end of the construct (base 1790) corresponding entirely to translated regions. I subcloned this into Bluescript II cut at *Eco* RI and *Bam* HI (Fig. 2; *Bam* HI-cut ends are compatible with *Bgl* II ligation). Since the insert was only compatible with

insertion in one direction, it was not necessary to determine the orientiation. Transcription with T3 was predicted to give the anti-sense.

2.7.3.1.5 β-actin

As a control, ubiquitously expressed target (though weak in keratinocyte suprabasal layers), a probe for β-actin was obtained through Stuart Naylor, ICRF from Derek Gatherer (Gunning *et al*, 1983), consisting of a 700bp *Pst*I-*Ava*II insert cloned into Bluescribe (Stratagene; Fig. 2). The insert represented a small 5′ untranslated portion and the first ~650 bp of the 5′ exon. Cutting with *Hind* III yielded a T7 transcribable fragment which produced the anti-sense strand; cutting with *Eco* RI yielded a T3 transcribable fragment which produced the sense strand.

2.7.3.2 Probe synthesis

Probes subcloned as described above, into pBluescript II were transcribed as follows. All pBluescript II-cloned probes were removed as a cassette by digesting with *Bss* HII (Stratagene), yielding the cDNA probe flanked by T3 and T7 bacteriophage promoters. It was not necessary to separate the cassette from the vector sequences, as transcription could only be initiated within the cassette. Other probes (e.g. β-actin) were prepared by cutting at a restriction site downstream from the promoter to be used for initiating transcripts. All digests were purified by two extractions with phenol/chloroform, followed by chloroform; or by three extractions with Strataclean resin (Stratagene). Final concentration of cut probe was 1mg/ml.

2.7.3.2.1 Transcription

A transcription kit (Stratagene), including T3 and T7 RNA polymerases, was used to generate ^{35}S-labeled (Amersham International, UK; SJ40383 ^{35}S-UTP, 40mCi/ml, specific activity 800Ci/mmol) probes. All components, except polymerases, were thawed on ice, then allowed to warm to near room temperature. Reactions were not set up on ice, as the

spermidine in the transcription buffer can precipitate DNA in the cold. These reaction conditions gave primarily complete transcripts as determined by urea polyacrylamide gel analysis (see Fig. 3). Control (sense) transcriptions were also included. The following components were set up the reaction in the order indicated:

0.5-1µg restricted, purified DNA	1 µl	
DEPC ddW	2 µl	
5x transcription buffer	3 µl	
10mM ATP, GTP, CTP	1 µl each	
0.5 M DTT	1 µl	
RNase inhibitor	1 µl	
^{35}S-UTP, 40mCi/ml, >800Ci/mmol	3 µl	
10 U T3 or T7 RNA pol	1 µl	of 1/10 dilution of 50U/µl
	15 µl	

This was incubated at 37°C for 1-2 hours. During the last 15 minutes, 2 units of RNase-free DNase were added, to remove template and therefore prevent its hybridization to target sequences.

2.7.3.2.2 Transcript purification

To remove the transcript from the unincorporated nucleotide, the reaction mix was passed over a Sephadex G-50 spin column (Clontech Laboratories Inc., Palo Alto, CA), following the manufacturer's method. The reaction mixture was brough to 25µl; 1 µl was removed for scintillation counting to determine original amount of label added. Following removal of unincorporated nucleotide, a further 1 µl was removed for counting and and the rest was frozen at -20°C immediately. The unpurified and purified transcripts were counted and the percent incorporation determined. It was at least 50%, and the yield of transcript was >10^8 cpm/µg DNA added. Transcripts were routinely analyzed on denaturing urea polyacrylamide gels (Fig. 3) for size and proportion of full length transcripts.

2.7.3.3 PAGE of RNA transcripts

RNA transcripts generated by transcription of probes were separated on an 8M urea, 15% polyacrylamide gel to resolve transcripts (Fig. 3). ^{35}S-labeled RNA markers, synthesized from a kit (Gibco) and yielding transcripts of 9.5, 7.5, 4.4, 2.4, 1.35, and 0.78kB were used to size full-length transcripts. It was necessary to achieve minimum gel thickness; a vertical mini-gel apparatus with 0.5mm spacers gave the best results (Mighty Small II, Hoefer). The apparatus was assembled according to the manufacturer's directions and the following solution was freshly prepared and poured: 8M electrophoresis grade urea (Bio-Rad), 15% electrophoresis grade acrylamide (40% liquid stock; BDH), 0.25% bis-acrylamide (Bio-Rad), made up in Millipore-filtered water. Just before pouring, TEMED (10µl/50ml; Bio-Rad) and ammonium persulfate (Bio-Rad) to 1% were added to initiate polymerization. A 15-well comb was inserted directly into the resolving gel; no stacking gel was used. Polymerization was allowed to proceed for no more than 1 hour since thereafter the urea started to crystallize, reducing the volume of the gel and eventually eliminating the wells.

0.5 x 10^6 cpm of RNA transcript was denatured as follows: the volume of RNA was brought to 3µl with DEPC water, and 4µl gel loading buffer (85% deionized formamide; 5% DEPC water; 5% 20X TBE; and 5% of a 10% xylene cyanol solution (Sambrook *et al*, 1989)) was added. Samples were denatured at 100°C for 4 minutes and immediately added to wells. Before addition of the samples, the gel was pre-run at 15 watts to heat the gel; just before sample loading, the wells were flushed with 1X TBE to remove accumulated urea. Gels were run (as hot as possible, to help keep the RNA denatured) at 20 watts, constant wattage and ~450V. Gels were stopped when the xylene cyanol, migrating at about 150bp (Sambrook *et al*, 1989) was about 1cm from the bottom.

The gel was removed from the plates and fixed 15 minutes to one hour in 10% methanol, 5% acetic acid. The gel was dried at 80° C for one hour under vacuum on a gel drier. The dried gel was exposed to XAR-5 film (Kodak) for 2 hours at -70°C or overnight at room temperature. Generally, the majority of transcripts were full-length, and of the expected maximum length, with numerous minor partial transcripts.

2.7.3.4 Tissue/cell preparation

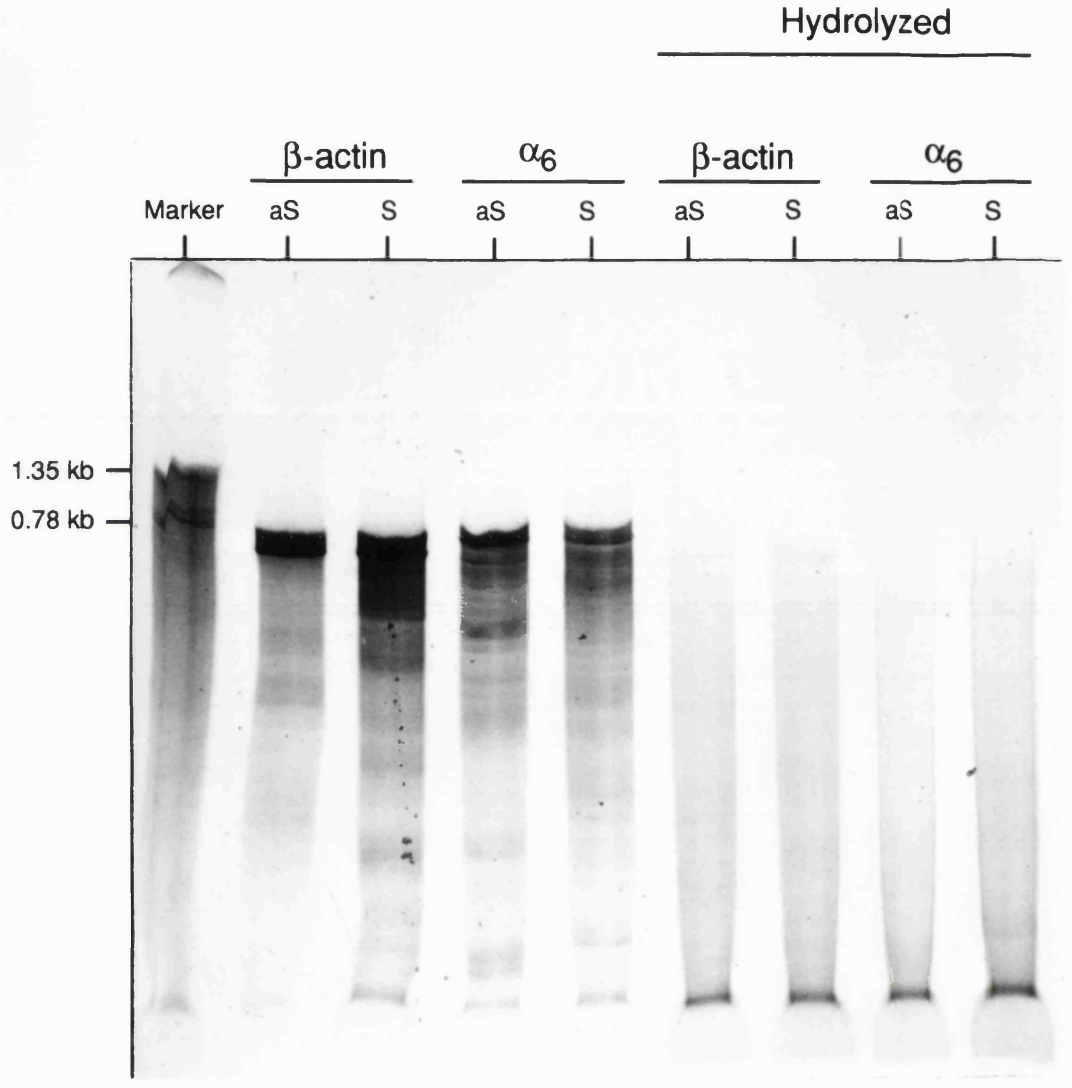
Tissues were fixed for 1 hour in freshly prepared 4% paraformaldehyde in PBS, washed 3 x 5 minutes in 0.1M glycine in PBS, coated in OCT (BDH) compound, and frozen in an isopentane/liquid nitrogen cooled to -70°C. 6 µm sections were cut onto baked, 1% gelatin/0.1% chrome alum coated slides, or onto silane-treated slides (Rentrop *et al*, 1986), although silane treatment was not as effective in ensuring section retention throughout the hybridization procedure. Gloves were worn during sectioning, and the knife was cleaned (e.g. with absolute ethanol) to prevent contamination of the sections with RNase. Sections were dried onto the slides and stored at -70°C. Hybridization was poor on tissue that had not been fixed before sectioning (not shown). Both cold acetone/methanol and paraformaldehyde fixation were tested for optimum target preservation in cultured cells; paraformaldehyde gave the best results. Cells were fixed for 30 minutes in 4% paraformaldehyde in PBS at room temperature, washed 3 x 5 minutes in 0.1M glycine, and either treated as for sections (below), or dehydrated through ethanol for storage at room temperature in a slide box containing desiccant.

2.7.3.5 Pre-hybridization treatment

Slides were allowed to warm up from -70°C to room temperature. Treatment for tissue or cells was identical, however, tissue slides were fixed again for 30 minutes in 4% paraformaldehyde before pre-hybridization treatment. Treatments were as follows:

a) 1 µg/ml proteinase K (Boehringer Mannheim, Germany) in 100mM Tris pH8, 50 mM EDTA pH 8 for 30 mins. at 37°C
b) post-fixation for 3 minutes in 4% paraformaldehyde
c) 3 x 5 minute washes in 0.1M glycine wash
d) acetylation: slides were placed in a rack, raised above the bottom of the dish with two blue pipette tips. 0.1M triethanolamine, pH 8.0, in 0.9% NaCl and a small stir bar were added

Figure 3 Autoradiography of radioactively labeled *in vitro* RNA transcripts separated by polyacrylamide gel electrophoresis.



aS = anti-sense transcription
S = sense

while stirring fairly vigorously, acetic anhydride (BDH) was added to 0.25% final
stirring was continued for 10 minutes

e) slides were rinsed twice in DEPC distilled water
f) slides were taken once through 70%, 95%, and twice through 100% ethanol for 1 minute each to dehydrate
g) slides were air-dried and stored in a slide box, desiccated and dust-free.

2.7.3.6 Hybridization

The hybridization solution was as follows:

50% deionized formamide
4X SSPE
125 µg/ml yeast poly A RNA
250 µg/ml yeast tRNA
1X Denhardt's
10% dextran sulfate (MW=500,000)

This was aliquoted to 500 µl and stored at -70°C.

The slides were placed in a chamber humidified with 5X SSPE. The probe was diluted in warmed hybridization buffer so that 1-2 x 10^6 cpm per section in a total volume of 14 µl could be applied. DTT was then added to a final concentration of 100mM and 15µl of mix was added to the center of each section. Control sections to which only the hybridization buffer and no probe were added were included. Baked coverslips were cleaned with 100% ethanol, air dried, and carefully laid onto the section, being careful not to trap any air bubbles. The incubation chamber was sealed with tape and incubated at 50° C for 16-24 hours.

2.7.3.7 Washing

Coverslips were removed and discarded, and the slides were placed into a large staining dish containing 2X SSPE/0.1% SDS. This was poured off and then the following washes were used: four 5 minute washes in 2X SSPE/0.1% SDS at room temperature, shaking gently; a 2X SSPE rinse; 30 minutes in 2X SSC + 10 µg/ml RNase A; two 30 minute washes in 0.1X SSPE/0.1% SDS at 60° C. Slides were then dehydrated through alcohols: twice in 70% ethanol for 1 minute; once in 95% ethanol for 1 minute; and twice in 100% ethanol for 1 minute.

Slides were allowed to air-dry thoroughly; usually slides were immediately coated with emulsion, but they could be stored until dipping.

2.7.3.8 Slide coating and exposure

Slides were coated with photographic emulsion to detect hybridized probe. Kodak NTB-2 emulsion, melted at 42°C, was diluted 1:1 with sterile distilled water, aliquoted, and stored in a light-tight box at 4°C up to 6 months. Liquid emulsion could be exposed to very low levels of dark room safe light, but dried emulsion was handled in total darkness. An emulsion aliquot was melted in the dark room for 45 minutes. Emulsion was gently poured into a two-slide mailer and hybridized slides were dipped in slowly, removed, and dipped again. Two plain, unhybridized control slides were also dipped. Slides were allowed to drain upright. Once dipped, the slides were allowed to air dry in total darkness for about an hour, then put in a slide box with fresh desiccant and sealed with tape, then wrapped with aluminum foil. Slides were exposed at 4°C for 2-8 days.

2.7.3.9 Slide developing

Slides were developed in Kodak D-19 or Dektol developer, diluted 1:1 or 1:3 with water. The developer and fixer were at the same temperature, and less than 20°C. The slide box was allowed to warm up to room temperature and a control, unhybridized slide was removed and exposed

briefly to room light. The other control slide was then removed in absolute darkness and both were developed. The light-exposed slide (which turned black when developed) checked solution strength, and the other slide (which was clear, i.e. unexposed when developed) controlled for light leakage during exposure or pre-exposure of the emulsion. Slides were developed for 4 or 2.5 minutes with occasional gentle agitation in absolute darkness, then rinsed briefly in tap water, then fixed for 2.5 minutes in Kodak hardening fixer, again with occasional agitation. Slides were rinsed for at least 20 minutes in tap water, followed by several brief rinses in distilled water, allowed to dry and stored dust-free. Slides were counterstained in Giemsa for 4 minutes and viewed under dark field to visualize grains.

2.7.4 Agarose mini-gel electrophoresis

To analyze the size and restriction fragment pattern of DNA, 1% agarose mini-gels were run. Electrophoresis-grade agarose (Gibco) was melted in 1X TAE, cooled to ~55° C, and poured into a mini-gel apparatus (Pharmacia-LKB 2013 Miniphor). Once the gel had set, 1X TAE buffer, containing 20-50µl of 10mg/ml ethidium bromide, was added to just cover the gel. DNA to be analyzed was loaded at 0.5-1µg per lane, with dye-containing loading buffer added (10% Ficoll, 0.025% bromophenol blue (Sambrook *et al*, 1989)). Gels were run at 80mA constant current for 60-90 minutes, and results were documented with Polaroid photography of stained DNA on a 302nm wavelength UV light box.

2.7.5 Electroelution of DNA fragments from agarose gels

Two methods were used for electroelution: originally, drawing the fragment out of an agarose gel piece into dialysis tubing; and later using an electroelution device (IBI Model UEA, New Haven, CT) which offered greater convenience.

2.7.5.1 Elution into dialysis tubing

The piece of agarose containing the fragment to be isolated was cut out under UV light and placed into a piece of dialysis tubing (molecular weight cutoff 6-8000; Spectra/Por, Los Angeles, CA). 200µl of $^1/_2$X TAE buffer was added to the bag and it was clipped closed, eliminating as much air as possible. The piece of agarose was pushed to one side of the tubing and the tubing was placed into a horizontal electrophoresis apparatus (GIBCO-BRL Model H5), with the agarose towards the anode, so that the DNA would run out of the agarose towards the cathode and therefore into the space in the bag. 100V, at constant voltage, was applied for one hour. The liquid was then removed from the dialysis bag and the bag was rinsed with a further 200µl $^1/_2$X TAE. The fragment was precipitated as described above and resuspended in a small volume of TE, pH8.

2.7.5.2 Elution using IBI apparatus

Agarose containing the DNA to be isolated was cut out as above and the DNA was electroeluted according to the manufacturer's instructions. The electroelution apparatus was soaked in 1M hydrochloric acid to destroy any DNA remaining from previous electroelutions and rinsed in sterile PBS. The agarose was placed into the wells and running buffer (20mM Tris-HCl, pH 8.0, 0.2mM EDTA, and 5mM NaCl) was added to the apparatus. The V-trap wells were cleared of air bubbles and 75µl of high salt buffer (7.5M sodium acetate with 0.01% bromophenol blue added to make it visible) were added to the V-trap. Run at 125V for one hour, DNA was drawn out of the agarose and into the salt cushion, from which it cannot escape. 400 µl was removed from the V-trap, 100% ethanol was added and the DNA recovered as previously described. Recovery of DNA averaged 60%.

2.7.6 DNA ligation

DNA ligations were carried essentially as described in (Sambrook *et al*, 1989). Insert and vector DNA were digested to have compatible termini. 100ng of vector DNA and an equal amount of insert DNA were placed in a

microfuge tube with sterile distilled water to a total volume of 7.5 µl. The solution was heated to 45° C for 5 minutes to dissociate any cohesive termini, and chilled on ice. Then 1 µl of T4 DNA ligase buffer, containing ATP (Gibco-BRL), 0.1 Weiss Unit of T4 DNA ligase (Gibco-BRL) and water to a total volume of 10 µl were added. The reaction was allowed to proceed at 16° C for 4-16 hours.

Control ligations, using vector DNA or insert DNA alone, were also routinely included. Following ligation, bacteria were transformed as described below.

2.7.7 Transformation of competent bacteria with plasmid constructs

Plasmid constructs were introduced into competent *E. coli* to amplify and propagate them. The XL1-BLUE strain of transformation-competent *E. coli* (Stratagene), which supports blue-white color selection of pBluescript II plasmids containing inserts, was exclusively used. The manufacturer's instructions were followed explicitly. Competent cells, stored at -70°C, were thawed on ice, mixed gently, and aliquoted to 100µl in pre-cooled Falcon 2059 polypropylene tubes. To each aliquot, 1.7µl of a fresh 1:10 dilution of 14.4M stock β-mercaptoethanol was added, giving a 25mM final concentration. Tubes were swirled gently, then swirled again every two minutes for a total of 10 minutes. 50ng of DNA to be transformed (or ligation controls) was added and the tube swirled gently. Additionally, 0.1ng of pBR322 DNA included in the kit as a positive control was added to an aliquot to test the efficiency of transformation. Tubes were incubated on ice 30 minutes, then heat shocked in a 42° C water bath for exactly 45 seconds, and placed on ice for two minutes. 0.9 ml of SOC medium (above) pre-warmed to 42° C was added to each tube and tubes were incubated at 37° C with agitation for one hour.

Transformed bacteria were then plated onto LB agar plates containing 50µg/ml ampicillin, which had been coated with 40µl of 100mM IPTG (isopropylthio-β-D-galactoside; Gibco) and 100µl of 2% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Gibco) to allow color selection. Plates with 10, 50, or 200µl of transformed cells were prepared and incubated overnight at 37° C. Any colonies which appeared were ampicillin-

resistant (excluding satellites) and had therefore received the pBluescript II; blue colonies had not had their lac Z gene interrupted by insertion of the foreign DNA and therefore could convert the IPTG/X-gal substrate to blue; white colonies were unable to convert substrate and these were individually picked and grown up for further analysis.

2.7.8 Plasmid isolation by alkaline lysis

2.7.8.1 Maxi-prep

This method is primarily derived from (Sambrook *et al*, 1989) and was used when milligram quantities of plasmid were needed. A scrape from a glycerol stock or a picked colony of bacteria containing the plasmid to be isolated was grown for 4-8 hours at 37 °C with vigorous rotation in 5-30ml LB broth + antibiotic appropriate to the plasmid. LB consisted of 10g Bacto-tryptone (DIFCO), 5g Bacto-yeast extract (DIFCO) and 10g NaCl made up to one liter with sterile distilled water and autoclaved. This formed the basic growth medium for all bacterial strains used. Antibiotics were added to LB: ampicillin to 50 μg/ml; tetracycline to 15 μg/ml; or kanamycin to 25 μg/ml. Once the small culture had grown to high density, it was added to 2 or 4 500-ml flasks, each containing 250 ml LB with antibiotic and incubated overnight.

All of the following quantities were for a 1000ml culture volume, and were halved for 500ml cultures. Bacteria were transferred to 500ml Nalgene bottles and spun down (30 minutes at 4000rpm; or 10 minutes at 6000; slower speed was preferable to keep pellet from packing too tightly) at 4°C. The supernatant was removed and the pellets resuspended in 30ml of 50mM glucose, 25mM Tris-Cl pH 8, and 10mM EDTA pH 8. Suspensions were pooled into one bottle and could be frozen at -20 °C at this point, if desired. Otherwise, 150mg of lysozyme (Sigma) was added to remove bacterial cell walls and the suspension swirled, then left at room temperature for 10 minutes. Then 40ml fresh 1% SDS, 0.2M NaOH was added to lyse the bacteria, the bottle was swirled and placed on ice 10 minutes, with occasional swirling. 30ml of potassium acetate solution (18ml 5M KAc, 3.45ml glacial HAc, and 8.55ml sterile distilled water) was added, mixed well, and placed on ice 10 minutes to neutralize the base and

precipitate bacterial membranes and trapped DNA. This was centrifuged for 20 minutes at 10,000 rpm and 4°C. The supernatant, containing small DNA molecules, was transferred to a 250ml centrifuge bottle, and 0.6 volume of isopropanol was added and left at room temperature for 30 minutes, to precipitate plasmid DNA.

DNA was pelleted by centrifugation for 20 minutes at 10,000 rpm and 4°C. The supernatant was discarded and the pellet rinsed three times with 70% ethanol. The bottle was inverted and the pellet allowed to dry almost to completion. The pellet was dissolved into 8ml TE, pH 8.0. 5 µl was removed for analysis on an agarose gel to check presence of the plasmid, and the rest purified on a cesium chloride gradient, or the following method was used if the crude preparation was fairly clean and further subcloning and purification were to be performed. RNase A (boiled to destroy DNase; Sigma) was added to 0.02mg/ml, the DNA was vortexed, and incubated 15 minutes at 37 °C to digest RNA. This was then phenol-chloroform extracted and precipitated, then resuspended in 500-1000µl TE pH8. Quantitation by optical density (Sambrook *et al*, 1989) and gel electrophoresis were performed. Additionally, a diagnostic restriction enzyme digest was used to confirm identity of the plasmid.

If greater purity was necessary, then a cesium chloride (CsCl) density gradient was run, to isolate pure, supercoiled plasmid. The volume was brought to 9.5ml and molecular biology grade CsCl was added at 1.0g/ml, i.e. 9.50 grams, and dissolved. 800µl of 10mg/ml ethidium bromide (EtBr) was added to stain the DNA and increase buoyancy of nicked DNA, and the solution was centrifuged 5 minutes at 2500rpm to pellet the EtBr/protein complexes that formed. The supernatant was transferred to an autoclaved SW70.1 polypropylene tube (Beckman) and topped up with 1.0g/ml CsCl in TE. Tubes were sealed and centrifuged in a 45° fixed-angle 70.1Ti rotor (Beckman) at 60,000rpm for 21 hours at 20°C.

The tube was removed and examined first in room light. Two bands were visible, unless the amount of DNA was small. The upper band was nicked plasmid and genomic fragments (which have bound more EtBr and were therefore more buoyant), the lower one was supercoiled plasmid (which is topologically constrained from binding as much EtBr as nicked). The lower one only was collected. If the bands were faint, DNA was collected

under UV, minimizing UV exposure, as the DNA would be nicked when EtBr is bound. The side of the tube was cleaned with 70% ethanol, dried, and a piece of Scotch tape was stuck over the area of the bands. The top of the tube was pierced with a needle, and then the side of the tube was pierced just below the band, with a 19g needle (bevel up) on a 2ml syringe. The band was carefully withdrawn and transferred to a polypropylene tube. The EtBr was extracted with an equal volume of isopropanol that had been saturated with a solution of TE which had itself been saturated with CsCl, by vigorous shaking. The upper, organic layer was red with the lower, aqueous layer slightly pink. The layers were allowed to separate and the upper layer was removed. Extraction was repeat 5-6 times until the aqueous layer was no longer pink, centrifuging on the last time to ensure separation. The plasmid solution was dialyzed against several changes of TE over several hours to remove the CsCl, and 1/10 volume of NaAc, pH 4.8 added before adding 2.5 volumes of absolute EtOH, mixed, and placed at -20 C overnight. DNA was recovered by centrifuging the precipitate at 10,000 rpm for 30 minutes at 4°C. The pellet was washed 3 times in 70% ethanol, air dried, and resuspended in 500-1000 µl TE pH8.

2.7.8.2 Mini-preps

Two methods were used. The classic alkaline lysis method, with modifications suggested by Stratagene, was used most of the time. Later, a method (Magic mini-preps; Promega) which used alkaline lysis followed by proprietary DNA binding beads and DNA elution was used as it was significantly faster and cleaner.

2.7.8.2.1 Alkaline lysis

The method used, a simplification of that above for maxi-preps, is originally detailed in (Sambrook *et al*, 1989), with modifications by Stratagene which sped up the procedure. 1.5ml of bacteria was spun at 13,000 rpm and the supernatant discarded. The pellet was resuspended in 100 µl ice-cold 50mM Tris-Cl pH 8.0, 10mM EDTA pH 8.0, then 200 µl 0.2M NaOH, 1% SDS was added. The tube was inverted several times and placed on ice for 5 minutes. 150 µl ice-cold potassium acetate solution (as

above for maxi-preps) was added and the tube vortexed. The tube was centrifuged for 5 minutes at 4°C, 13000 rpm; the supernatant was transferred to a fresh tube. 20 µl of Strataclean resin (Stratagene) was added, the tube was vortexed, and incubated one minute at room temperature (Strataclean substitutes for phenol/chloroform, to remove proteins). The tube was centrifuged at 13000 rpm for 2 minutes and the supernatant was transferred to a fresh tube. Two volumes of absolute ethanol at room temperature were added, the tube was vortexed, and then incubated at room temperature for 2 minutes. The DNA was pelleted by centrifuging the tube at 13000 rpm for 5 minutes and the ethanol was allowed to drain out of the inverted tube. The pellet was allowed to dry almost to completion, then resuspended in 10 µl TE.

2.7.8.2.2 DNA affinity beads (Magic™)

The manufacturer's protocol was used. Briefly, bacteria were lysed with reagents provided, in a modification of the method described above. Supernatant containing plasmid DNA was allowed to bind to beads which were then spun in a micro-column and then washed. Plasmid DNA was eluted with distilled water at 70° C; recoveries from 1.5ml of bacteria averaged 10µg.

2.7.9 RNA extraction

RNA was extracted using the guanidine isothiocyanate method (Sambrook *et al*, 1989), or the RNAzol method (Chomczynski and Sacchi 1987), which was more rapid.

2.7.9.1 Guanidine isothiocyanate (GITC) method

The guanidine isothiocyanate method, originally described by (Chirgwin *et al*, 1979) was followed as described in (Sambrook *et al*, 1989). Feeder cells were removed from keratinocyte cultures, using versene, and keratinocytes were lysed in the following solution: 50g guanidine isothiocyanate; 0.5g N-lauroylsarcosine (sodium salt); 2.5ml 1M trisodium

citrate, pH 7.0; and 0.33ml Antifoam A were dissolved in and brought to a total volume of 100ml, filter sterilized, and 700µl 14.4M β-mercapto-ethanol was added. Aliquots were stored at -20° C until needed. RNA lysates were passed through a 23g needle to shear cellular DNA and brought to a total volume of 8ml in GITC.

Samples were stored at -20°C, or immediately layered onto a 4 ml cesium chloride gradient (5.7M CsCl, 25mM sodium acetate, pH 6.0) and centrifuged in an SW40.1 rotor (Beckman) at 32,000 rpm for 21 hours. Supernatant was discarded and the RNA pellet was washed twice in 70% ethanol, then air-dried until it had a jelly-like appearance. Pellets were resuspended in 10-100µl TE pH 7.6, quantitated by spectrophotometry (Sambrook *et al*, 1989) at 260/280nm, and stored aliquoted at -70° C.

2.7.9.2 RNAzol method

Feeders were removed and the keratinocytes trypsinized and pelleted. Pellets were completely resuspended in 200µl RNAzol B (Biotecx Laboratories, Inc., Houston TX) per 10^6 cells. Alternatively, 4 ml of RNAzol could be added directly to the flask. 1/10th volume of chloroform was added and the samples were vortexed for 15 seconds, followed by five minutes on ice. The suspension was microfuged at 13, 000 x g at 4° C for 15 minutes and the aqueous phase was transferred to another tube containing an equal volume of isopropanol. RNA was precipitated on ice for 15-60 minutes and centrifuged at 13, 000 x g at 4° C for 15 minutes. Pellets were washed in 70% ethanol, dried, and resuspended in TE, pH 7.6. Aliquots were stored at -70° C.

2.7.10 Northern Blotting

RNA was separated on a formaldehyde denaturing gel, based on the method in (Sambrook *et al*, 1989 and Nicholson and Watt 1991). A horizontal electrophoresis tank (Gibco-BRL Model H5) was treated for 10-30 minutes in 1M HCl to destroy nucleases and rinsed in DEPC-treated PBS, then DEPC-treated water. Gels, consisting of 7.4% formaldehyde, 1X Hepes buffer (0.05M Hepes, 1mM EDTA, pH 7.9), and 1% agarose in DEPC-

treated water, were poured in a fume hood and allowed to gel 45 minutes. RNA samples were prepared by adding 15µg RNA in a volume of 4µl, to 4µl formaldehyde, 10µl deionized formamide, and 2µl 10X Hepes buffer. RNA marker (4 µg; sizes as for RNA PAGE; Gibco-BRL) was treated identically, as was 5µg of total keratinocyte RNA to use as an 18S/28S size marker. RNA was denatured by heating to 70°C for 10 minutes. 2 µl of loading buffer (50% glycerol, 50% 10X Hepes, 0.25% bromophenol blue) was added to each sample, and samples were loaded on the gel which had been submerged to 1mm depth in 1X Hepes running buffer. The gel was run at 100V constant voltage until the dye had migrated 2/3 of the way to the end, about 4 hours. The marker ladder and RNA lanes were cut off and stained for one hour in 10µg/ml ethidium bromide, followed by an overnight destain and photography. The gel was inverted and placed into a capillary transfer set-up (below) for transfer to nylon membrane.

2.7.11 Probes used for Northern blot hybridization

The following DNA probes were radioactively labeled (below) and used to hybridize to immobilized target RNA or DNA.

2.7.11.1 β_1

The insert or whole plasmid from pFNRβ (also called λP32, (Argraves *et al*, 1987), coding for the entire human β_1 integrin subunit and a 1117bp 3' untranslated region, was generously donated by E. Ruoslahti, La Jolla Cancer Research Foundation, La Jolla, CA.

2.7.11.2 β-actin

The same cDNA as described above for *in situ* hybridization was labeled and used (Gunning *et al*, 1983), generously donated by D. Gatherer.

2.7.11.3 18S

18S rRNA, ubiquitously expressed, was used as a loading control target, and probed with plasmid 100 D9, coding for mouse 18S (Edwards *et al*, 1987); generously donated by D. Edwards, University of Oxford.

2.7.11.4 Involucrin

Involucrin expression was analyzed using a probe to human involucrin, pI-2 (Eckert and Green 1986), generously donated by Howard Green, Harvard Medical School, Boston MA.

2.7.11.5 ICAM-1

ICAM-1 expression was detected with a probe to mouse ICAM-1 (Simmons *et al*, 1988), generously donated by David Simmons, ICRF.

2.7.12 Genomic DNA extraction

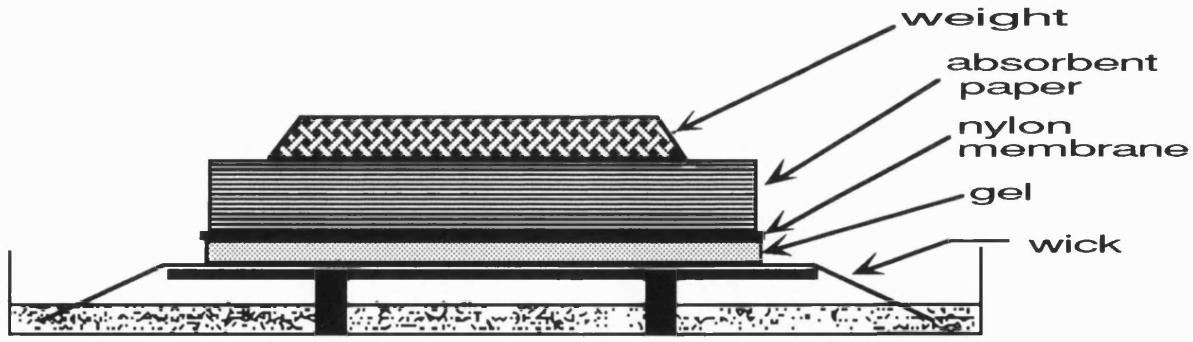
This method is essentially from (Sambrook *et al*, 1989). Feeders were removed and ES cells from 2-3 T75s were trypsinized as described above. Pellets were stored at -20° C, or processed immediately. Pellets were resuspended in 5ml TE pH 8, proteinase K (Boehringer Mannheim) was added to 200µg/ml, and the solution was mixed well. SDS to 1% was added, the solution was mixed and incubated at 37° C overnight. Then, an equal volume of equilibrated phenol was added and the sample placed on a rotating wheel inverter for 10 minutes. The layers were separated by centrifugation at 1500rpm for 10 minutes. A 10ml disposable plastic pipette, with the tip broken off to minimize DNA shearing, was used to draw off the aqueous layer, which was extracted again with phenol. The aqueous phase was then extracted with chloroform, and NaCl was added to 0.3M, followed by two volumes of cold absolute ethanol. Generally, DNA precipitated into a fibrous mass which could be spooled out. Otherwise, the sample was centrifuged for 30 minutes at 10,000rpm. Pellets/spooled material were washed with 70% ethanol, air dried, and resuspended in 50-100µl TE pH 8.

2.7.13 Southern Blotting

10µg of DNA was run in a 1.2% agarose horizontal gel apparatus (Gibco-BRL Model H5) buffered with TAE, as detailed for minigels. The gel was stained with ethidium bromide and photographed, then depurinated with 0.25M HCl for 15 minutes and rinsed in distilled water (Sambrook *et al*, 1989). The DNA was then denatured with 0.2M NaOH/0.6M NaCl for 30-60 minutes, then rinsed in distilled water. The gel was neutralized in 0.5M Tris/1.5M NaCl, pH 7.5 twice for 30 minutes each, then transferred to a capillary transfer setup as detailed below.

2.7.14 Capillary transfer

Capillary transfer was performed as described in (Sambrook *et al*, 1989). As illustrated below, a wick, submerged in 20X SSC, was set up so that the SSC was drawn through the gel, carrying the nucleic acid into the nylon membrane (0.45µm Hybond-N; Amersham) where it was immobilized. The SSC continued into a stack of paper towels cut to the size of the blot. Transfer was allowed to proceed overnight. Blots were rinsed in 2X SSC to remove excess salt and baked at 80° C for two hours to fix the nucleic acid to the membrane. Dry membranes were stored dark and cool until hybridization.



2.7.15 Random primer extension

DNA probes were labeled using random primer extension, which uses the Klenow fragment of DNA polymerase to generate radioactively labeled

DNA, primed by random hexamers bound to denatured probe DNA. A kit (Multiprime; Amersham) was used to label 50ng of DNA (usually insert specific for the sequences to be probed) denatured at 100° C for 5 minutes, using 50μCi of ^{32}P-dCTP (specific activity 3000Ci/mmol; Amersham # PB10205, UK). Reactions were allowed to proceed at 37° C for one hour, and unincorporated nucleotides were removed (below). A kit which used nonamers (Megaprime; Amersham) was also used; labeling conditions were identical except that labeling took 5 minutes.

2.7.16 Removal of unincorporated nucleotides from labeled probes

Following transcription or random primer synthesis of RNA or DNA probes, unincorporated nucleotides were removed either by using a gravity-fed Sephadex G-50 drip column (Pharmacia), or G-50 spin columns (Clontech); in both cases, the manufacturer's method was followed.

2.7.17 Filter hybridization

Hybridization of radioactive probes to either RNA or DNA immobilized on nylon membranes was performed as follows. At first, hybridization was carried out in sealed PVC bags; later, a hybridization roller oven, which ensured complete coverage of the membrane (Techne HB-1) was used. Filters were pre-hybridized for 1-4 hours in 5X Denhardt's, 50% deionized formamide, 5X SSPE, 1% SDS, and 200μg/ml denatured, sonicated salmon sperm DNA at 42°C (Sambrook *et al*, 1989). The solution was exchanged for fresh, pre-warmed hybridization buffer and radioactively labeled probe (25-50ng at high specific activity) was added. Hybridization was allowed to proceed overnight at 42° C with agitation or rolling. The hybridization buffer was poured off, and the blot rinsed in 2X SSPE/0.1% SDS, then washed twice in 2X SSPE/0.1% SDS for 30 minutes each at 42° C. The blot was rinsed in 0.1X SSPE/0.1% SDS, then washed 30 minutes in pre-warmed 0.1X SSPE/0.1% SDS at 60-65° C. If background counts, as judged with a Geiger monitor, were still high (>100cpm) where hybridization was not anticipated, the blot was washed for a further 30 minutes. Excess liquid was removed from the blot by dabbing with 3MM paper. The damp blot was wrapped in cling film and placed between XAR-

5 film (Kodak) and a phosphorescent enhancing screen (DuPont). Initial exposure was for one day at -70°C, with a longer exposure if necessary. Exposure for involucrin or 18S RNA was on the order of hours.

2.7.18 Densitometry

A laser scanning densitometer (Pharmacia-LKB Ultroscan XL) was used to quantitate band density for Northern blots, according to the manufacturer's instructions. The area of each band was normalized for loading by comparison to a housekeeping mRNA, e.g. actin, and results were normalized compared to control keratinocyte values.

2.8 Table 1. Integrin antibodies used and their sources

All the antibodies are mouse monoclonal, recognizing human integrins, unless otherwise noted. All antibodies are specific for the subunit indicated and are not heterodimer-dependent (Tenchini *et al*, 1993).

Antibody name	Antigen	Source and reference
TS2/7	subunit α_1	M. Hemler, Dana Farber Cancer Research Institute, Boston MA; (Hemler *et al*, 1984)
P1E5	α_2	E. Wayner, Oncogen Science, Inc., Seattle, WA; (Carter *et al*, 1990)
P1E6	α_2	Telios Pharmaceuticals, San Diego, CA
5E8	α_2	R. Bankert, Roswell Park Memorial Institute, Buffalo, NY; (Zylstra *et al*, 1986)
HAS-4, HAS-6	α_2	F.M. Watt, ICRF; (Tenchini *et al*, 1993).
J143	α_3	A. Albino, Sloan-Kettering Memorial Institute, New York, NY; (Kantor *et al*, 1987)
VM-2	α_3	American Type Culture Collection, (Morhenn *et al*, 1983)
B-5G10	α_4	M. Hemler; (Hemler *et al*, 1987)
mAb 16	α_5	Rat monoclonal; K. Yamada, NCI, Bethesda, MD; (Akiyama *et al*, 1989)
BIIGII	α_5	C. Damsky, Univ. of California, San Francisco; (Knudsen *et al*, 1981)
P1D6	α_v	Telios Pharmaceuticals, San Diego, CA.
GoH3	α_v	Rat monoclonal; A. Sonnenberg, University of Amsterdam, The Netherlands; (Sonnenberg *et al*, 1986)
VNR 147	α_v	Telios Pharmaceuticals, San Diego, CA.
13C2	α_v	M. Horton, ICRF, London; (Horton *et al*, 1985)
mAb 13	β_1	Rat monoclonal; K. Yamada, NCI, Bethesda, MD; (Akiyama *et al*, 1989)

CD29	β_1	Janssen Pharmaceutical, UK
Y2/51	β_3	D. Mason, John Radcliffe Hospital, Oxford; (von dem Borne *et al*, 1989)
439-9B	β_4	S. Kennel, Oak Ridge National Laboratory, Oak Ridge, TN; (Kennel *et al*, 1989)
FNR	$\alpha_5\beta_1$ fibronectin receptor	Rabbit polyclonal; Calbiochem #341641
3E1	β_4	E. Engvall, Scripps Clinic, La Jolla, CA; (Hessle *et al*, 1984; Ryynanen *et al*, 1991)

2.9 Table 2. Non-integrin antibodies used and their sources.

Antibody name	Antigen	Source and reference
LH10	Keratin 10	Leigh, I.M., Purkis, P.E., Whitehead, P., and Lane, E.B.; Monospecific monoclonal antibodies to keratin 1 carboxy terminal (synthetic peptide) and to keratin 10 as markers of epidermal differentiation. *Br. J. Dermatol.*, submitted
LP34	Keratins 10, 18, 5+14, 6+16	B. Lane, (Lane *et al*, 1985)
LE61	Keratin 18	B. Lane, (Lane *et al*, 1985)
LL025	Keratin 16	B. Lane et al, 1992 in preparation
DH1	Human involucrin	D. Hudson, (Dover and Watt 1987)
GP7	Keratin 9 (guinea pig)	H. Heid; (Moll *et al*, 1987)
HECD-1	E-cadherin	M. Takeichi; (Shimoyama *et al*, 1989)
	Collagen type IV	M.J. Warburton, (Warburton *et al*, 1982)
	Fibronectin	Calbiochem, Nottingham, UK
	Laminin	M.J. Warburton, (Warburton *et al*, 1982)
15.2	ICAM-1	N. Hogg, ICRF, (Dransfield *et al*, 1992a)

Chapter 3

Integrin expression during epidermal development *in vivo* and *in vitro*.

3.1 Introduction

In mature epidermis, the integrins are involved in maintaining the epidermal architecture (see Chapter 1). During the development of the epidermis, differentiating keratinocyte layers must be successively built up from a single ectodermally-derived basal layer (reviewed in (Holbrook 1991) and discussed below), and I was intrigued by the possibility that integrins might also be involved in establishing the epidermal architecture. There is correlative evidence that the integrins are involved in the morphogenesis of several other organs that have been examined to date (discussed below). Additional differences between mature and developing epidermis, such as generation of adnexal structures, rapid proliferation to cover the expanding surface of the fetus, and forming the interface between the fetus and an aqueous environment could also reflect changes in integrins.

3.1.1 Development of human skin

Mature skin consists of a primarily keratinocyte-populated epidermis anchored to a basement membrane, with an underlying dermal connective tissue containing nerves, blood vessels, and fibroblasts in a collagen-based three-dimensional matrix.

The epidermis, along with the nervous system and the cells of the neural crest (from which the melanocytes originate), is derived from one of the three primary germ layers, the ectoderm, which covers the surface of the gastrula (Alberts *et al*, 1989). By about 4 weeks estimated gestational age (EGA), the ectoderm has given rise to a layer of basal keratinocytes covered by a layer of periderm cells, surrounding the fetus. At around 9 weeks EGA, stratification begins, and the basal layer generates an intermediate keratinocyte layer (i.e. between the basal and periderm layers) through proliferation and upward cell migration. Additional suprabasal layers are added periodically until 20-24 weeks, when the periderm is sloughed off and all the morphologically recognizable epidermal layers present in mature skin can be identified.

3.1.1.1 Periderm

The periderm is a transient feature of primate epidermis providing, it is thought, primarily a protective function prior to formation of the stratum corneum at approximately 22-24 weeks EGA. Fetal epidermis is highly permeable and there is evidence for a transport function of the periderm, perhaps to maintain the correct fluid and salt balance between the fetus and the amniotic fluid. The periderm undergoes remarkable morphological changes characteristic of the gestational age; the surface area of the periderm cells increases with gestational age, primarily through the formation of microvilli and a large, central bleb (see Fig. 1F; four blebs are apparent) on each cell. Initially the periderm is as proliferative as the basal keratinocytes, but as development proceeds, the periderm cells flatten to cover the increasing area and the labeling index declines (Bickenbach and Holbrook 1986). As parakeratinized cells form, the periderm is sloughed off into the amniotic fluid — these are some of the cells that are typically sampled for developmental abnormalities during amniocentesis. The periderm expresses keratins characteristic of simple epithelia e.g. keratins 8 & 18 (van Muijen *et al*, 1987) — suggesting that the periderm maintains the phenotype of the primitive ectoderm cells from which it originated (Moll *et al*, 1982b) — and of non-keratinizing epithelia (keratin 4; (van Muijen *et al*, 1987)). The periderm also expresses other epithelial markers, including Peri-1 (Lane *et al*, 1987), originally thought to be periderm-specific but now believed to be characteristic of transport epithelia (Riddle 1985), and involucrin by 11 weeks EGA (Watt *et al*, 1989), well before it can be detected in the differentiated keratinocytes. Periderm cells also form cornified envelopes (Watt *et al*, 1989).

3.1.1.2 Development of the epidermis

The keratinocytes of the fetus undergo numerous changes during development. The dominant morphological event, and also the point when many keratinocyte-specific markers can first be detected, is the onset of stratification — the addition of suprabasal keratinocyte layers. This first occurs at ~9 weeks EGA, slightly earlier in the palm and sole. After the onset of stratification the epidermis is termed fetal, instead of embryonic (Holbrook 1991). The first fetal suprabasal keratinocytes express most of

the markers of adult keratinocytes of the first suprabasal layer (Fisher and Holbrook 1987).

The suprabasal keratinocytes must arise by division of the basal keratinocytes. Although proliferation in embryonic epidermis is slightly higher than in fetal (labeling index of 8.5% vs. 7%; (Bickenbach and Holbrook 1986)), there is no apparent proliferative burst when stratification begins. There may be a fundamental and remarkable difference in the mechanism of stratification during development, when compared to the stratification that occurs in the adult. During mouse epidermal development, from the onset of stratification until keratinization, the cleavage plane of the basal cells is parallel to the basement membrane, i.e. one daughter cell is deposited in the suprabasal layer while the other one remains in the basal layer (Smart 1970). This is in contrast to the plane of cleavage in the neonatal mouse (Iversen *et al*, 1968; Smart 1970) and adult human (Robin Dover, pers. comm.), when the plane of cleavage is perpendicular of the basal layer, resulting in a committed daughter basal cell which detaches and migrates upward.

Development of the epidermis continues with additional layers being added suprabasally (i.e. continued stratification). By 12 weeks EGA, the epidermis consists of at least 4 layers, and another morphogenetic event takes place. Depending on the body site, sweat gland and/or hair follicle primordia start to invade the dermis. The condensation of the mesenchyme at regular intervals anticipates the clustering of basal cells (pre-germs) which will bud into the dermis to ultimately form a hair follicle, bearing a sebaceous gland (Holbrook 1991). In the case of the eccrine sweat glands (which secrete sweat onto the skin surface), primary epidermal ridges — slight epidermal downgrowths (particularly apparent in Fig. 4C) — are the first sign. Sweat ducts (e.g. Fig. 3D) develop quickly; secretory coils are evident by 16 weeks in the sole (Hashimoto *et al*, 1966).

By about 20 weeks EGA, parakeratinized cells start to appear at the surface of the epithelium and the periderm starts sloughing off. By 22-24 weeks, fetal epidermis is essentially mature in its morphology, with recognizable spinous, granular, and cornified layers.

Although many markers of adult epidermis are first expressed around the onset of stratification, they are sometimes not organized or assembled into their mature structures until later (Holbrook *et al*, 1987). For example, although involucrin can be detected in the first suprabasal cells at stratification, it is not cross-linked into cornified envelopes until much later.

The keratins are the best-characterized markers of the dynamic nature of keratinocyte development. There is an intricate, orderly progression of keratin expression, initially from simple epithelial keratins, to keratins typical of stratified, and ultimately keratinizing epithelia (Moll *et al*, 1982b; Dale *et al*, 1985; van Muijen *et al*, 1987). Until stratification, only keratins 8 & 18, and 5 (but not its partner K14) are expressed; at stratification, keratins 1 & 10/11 are present in small amounts in intermediate cells, increasing dramatically at 12-14 weeks EGA (Moll *et al*, 1982b; Fisher and Holbrook 1987). Markers of commitment to stratification (expression of K5) and keratinization (expression of K1 & 10/11) are thus expressed well before the actual events at about 9 and 23 weeks EGA respectively (Dale *et al*, 1985). Keratins 4 and 13, characteristic of non-keratinizing epithelia (and therefore appropriate for epidermis at this stage)(Moll *et al*, 1982a), and keratin 19, characteristic of simple epithelia, are present until 20 weeks (Moll *et al*, 1990). Interfollicular keratinocytes, and the keratinocytes of the hair follicle and sweat gland, have different keratins, even though they all derive from the same original basal cell layer (Moll *et al*, 1982b). Already at 13 weeks, hair germs manifest a less complex keratin pattern different from that of interfollicular keratinocytes, that will be retained in the adult. Mature epidermal keratinocytes express significantly fewer keratins than during early development. There are apparently no fetal-specific keratins.

Bullous pemphigoid antigen (BPA), a component of assembled hemidesmosomes (Stanley *et al*, 1981b; Robledo *et al*, 1990), does not appear until the onset of stratification. It is initially found in a discontinous distribution along the basement membrane but subsequently BPA expression increases as more hemidesmosomes form.

There are some regional differences in epidermal development (Holbrook and Odland 1980). For example, palm and sole epidermis

stratify more quickly than that from other parts of the body. Although the number and type of adnexae varies with body site, the essential organization of the epidermis is very similar in different body sites.

3.1.1.3 Origin and advent of non-keratinocyte cells of the epidermis

Keratinocytes, although by far the most numerous, are not the only cells of the epidermis. Melanocytes, Merkel cells, and Langerhans cells migrate to the epidermis during fetal development or originate there and are present by 6 weeks EGA. They do not appear to have any direct influence on the development of the epidermis, although they release cytokines that may have effects on morphogenesis (Moll *et al*, 1986).

Melanocytes

Derived from the neural crest, melanocytes are present in all epidermal layers, until 13 weeks EGA, when they become restricted to the basal layer. Melanocytes, through their synthesis of melanin, give skin its characteristic pigmentation by the third to fifth month EGA. Mature melanocytes express the α_3, β_1 and α_6 integrin subunits in skin (Zambruno *et al*, 1991b); but in culture they express a large variety of integrins (α_1-$\alpha_6\beta_1$, and $\alpha_v\beta_3$ (Albelda *et al*, 1990)). $\alpha_7\beta_1$, expressed on melanoma cells but not on mature normal melanocytes (Kramer *et al*, 1991), has not been examined for possible fetal expression. I and others have not detected any overt heterogeneity in integrin expression in fetal basal cells of the epidermis, suggesting that melanocytes have at least those integrins expressed by keratinocytes (and possibly also $\alpha_4\beta_1$ (Iida *et al*, 1992), though I did not detect any).

Merkel cells

These neuroendocrine cells function as mechanoreceptors and may serve as a target for ingrowing nerves. They are present in the adnexae, and in palmar and plantar epidermis between sweat ducts. Although Merkel cells were originally thought to be derived from the neural crest, migrating to the epidermis via an unknown route, they are now thought

to originate from epidermal precursor cells (Moll *et al*, 1986; Moll *et al*, 1990).

Langerhans cells

Langerhans cells are derived from the bone marrow and migrate to the epidermis (Foster *et al*, 1986). They may play an immunologic role in the epidermis, possibly functioning as antigen presenting cells (Holbrook 1991). Langerhans cells have a heterogeneous pattern of integrin expression, subsets expressing α_1-α_6 to varying degrees (Le Varlet *et al*, 1991). There are regional differences in Langerhans cell localization: palm and sole epidermis appear to lose Langerhans cells after 18 weeks EGA (Fujita *et al*, 1991).

3.1.1.4 Formation of the basement membrane

The basement membrane — also called the basal lamina — separates the epidermis and dermis and forms the substrate for keratinocyte attachment. In the adult, the basement membrane may take part in the maintenance of epidermal architecture (e.g. maintenance of polarity), and regulating commitment to terminal differentiation (Adams and Watt 1989; Streuli *et al*, 1991). The components of the basement membrane originate from both keratinocytes and fibroblasts. A linear deposit of laminin, suggesting a basement membrane, can be detected as early as 4 weeks EGA (Holbrook 1991). Collagen types I, IV (Fine *et al*, 1984), and V, heparan sulfate proteoglycan (Horiguchi *et al*, 1990) and fibronectin (Fine *et al*, 1984) can be detected shortly thereafter, and a basal lamina is microscopically evident. After stratification, collagen type VII, which interacts with hemidesmosomes, can be detected at the BMZ, synthesized by the fibroblasts (Olsen *et al*, 1992). Initially, the BMZ is somewhat amorphous, but by about 11 weeks EGA a lamina lucida (clear zone) and lamina densa (dense zone) are detectable (Horiguchi *et al*, 1990), although their distinction may be an artifact of electron microscopy. Defects in attachment to the basement membrane can have catastrophic effects; homozygous junctional epidermolysis bullosa, for example, is generally fatal (discussed in (Watt and Hotchin 1992)).

3.1.1.5 Development of dermis

The dermis originates from the dermatome region of the somites — which in turn originate from the mesoderm — and from the mesenchyme near the epidermis (Alberts *et al*, 1989). The mature dermis consists of a complex, fibrous matrix, mainly populated by fibroblasts, with a network (or plexus) of blood vessels and nerves interlaced throughout supplying the dermis, epidermis and epidermal structures. Unlike the epidermis, though, development of the dermis is still not complete at birth. The most conspicuous developmental change is a transition from a mesenchyme which has a low fibrous matrix and high water content with stellate, process-connected cells to a connective tissue characterized by bundles of fibrils and more typically fibroblastic cells (Holbrook *et al*, 1987) after the onset of stratification. By 16 weeks EGA, the adult-type dermal morphology consisting of reticular (deep) and papillary dermis (in contact with the basement membrane) can be discriminated. The principal non-cellular component of the dermis is collagen, primarily collagen type I, which forms large fibrils, but also types III, V, and VI. During development, collagens III and V decline in proportion relative to the adult (Smith *et al*, 1986). Elastin appears relatively late, around 24 weeks EGA (Holbrook *et al*, 1987). Dermal areas enriched in tenascin indicate areas of epithelial invasion where adnexal structures will form (Bourdon and Ruoslahti 1989; Ekblom 1989).

Other structures of the dermis, such as nerves and blood vessels, although present very early, undergo rearrangement and increase in complexity throughout development. The dermal vasculature is first apparent as capillaries, present in the earliest specimens studied (5 weeks EGA) organized in a plane parallel to the epidermis. I noted α_2 staining in flat, tube-like structures parallel to the epidermis by 8 weeks (not shown) which presumably corresponded to capillaries (Lampugnani *et al*, 1991). The capillary beds organize into several plexuses, still parallel to the epidermis, reorganizing as epidermal structures intrude. The adult pattern is established by the 7th month for the large vessels, but the capillaries continue to rearrange and increase in number until the expansion in skin area slows down after birth.

The nerves of the dermis reach the basal layer of the epidermis by 8 weeks, ultimately withdrawing to the dermis (Hogg 1941) while maintaining contact with Merkel cells (Sinclair 1973). As early as 8.5 weeks, the fetus can respond to cutaneous stimuli through the nerves of the face; within the next few weeks, the palms and soles can respond to contact. Like the basic vascular pattern, the fundamental nerve pattern is established soon after the onset of epidermal stratification.

Fibroblasts express $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$ (Schiro *et al*, 1991), and several α_v integrins ($\alpha_v\beta_1$, β_3, plus a putative β_5 and an unidentified β (Bates *et al*, 1991)). $\alpha_2\beta_1$ is responsible for collagen contraction and reorganization (Klein *et al*, 1991; Schiro *et al*, 1991), important during wound healing. Developmental regulation of fibroblast integrin expression has not been reported nor did I observe any: the subunits I examined were present at all times.

3.1.2 Integrin participation in morphogenesis

Why do we think the integrins are likely to be involved in epidermal morphogenesis? To date, there has been much correlative evidence for a role of integrins in development, but less work in which unequivocal evidence has been obtained. The best evidence for a morphogenetic role comes from naturally occurring integrin mutations, and from integrin function-blocking experiments using *in vivo* models. Two mutations in *Drosophila* have turned out to involve defects in the *Drosophila* integrins (known as the PS1-PS3, or position-specific, antigens), both involving the inability of the developing wing epithelia to correctly appose. *Drosophila* have two integrins, each with a unique α subunit (PS1 and PS2) but shared β (PS3). The *myospheroid* mutation (MacKrell *et al*, 1988; Leptin *et al*, 1989) , named for the lack of muscle group attachment resulting in curled-up muscles, is caused by the absence of the common β subunit. Another phenotype of *myospheroid*, wing blistering, is also a feature of the *inflated* mutation, caused by a defect in the PS2 α regulatory gene, resulting in greatly reduced expression. Although both of these mutations are lethal when homozygous, clearly indicating the vital role of integrins, they nonetheless result in surprisingly few morphogenetic abnormalities given the wide distribution

of integrins, suggesting that other adhesion molecules can take over at least some integrin functions.

Another approach to the study of integrin function is to use peptides, which mimic the integrin binding site of several ligands: GRGDS (RGD: Arg-Gly-Asp) is the active site of the central cell-binding domain of fibronectin (Humphries 1990) and can thereby inhibit the function of several integrins. Peptides — due to their much smaller size — also have the advantage over antibody blocking of achieving greater penetration of the tissue. The specificity of RGD effects can be confirmed with an inactive RGE peptide. The accessibility of chicken embryos has made them popular for antibody or RGD perturbation of integrins. Early work, on the CSAT antigen — a chicken integrin recognizing fibronectin — indicated the role of integrins in neural crest cell migration. Addition of RGD peptides to chick embryos resulted in neural crest cells distributing to the wrong locations, using abnormal pathways (Boucaut *et al*, 1984; Bronner-Fraser 1985; Lallier and Bronner-Fraser 1991). *In vitro*, RGD peptides inhibit fibronectin-supported neural crest cell migration out of neural tube explants (Boucaut *et al*, 1984). Blastoderm cell spreading, on a fibronectin substrate, out of explanted chick blastoderms is temporarily inhibited by an RGD peptide (Lash *et al*, 1990). Perturbation of chick skin morphogenesis (in organ culture) with anti-β_1 inhibits epithelial-mesenchymal interactions (Jiang and Chuong 1992) — without which some structures of the epidermis, e.g. hair follicles, cannot develop.

Perturbation of integrins during amphibian gastrulation has been studied using antibody and RGD peptide inhibition. Blocking integrin-fibronectin interaction with either an RGD peptide (Boucaut *et al*, 1984) or an anti-C terminal β_1 antibody (Darribère *et al*, 1990) prevents gastrulation in *Pleurodeles waltlii* and is therefore lethal. Here, inhibition of integrins blocks cell migration, e.g. invagination into the blastopore, possibly because fibronectin fibrils are unable to assemble correctly without integrin participation (Boucaut *et al*, 1984). In the frog, *Xenopus laevis*, integrin-mediated spreading on fibronectin by animal pole cells induced to undergo gastrulation-like movements is inhibited by RGD peptides (Smith *et al*, 1990).

Roman *et al* have shown that RGD peptides, but not RGE, are able to specifically inhibit mouse lung epithelial branching morphogenesis in organ culture (Roman *et al*, 1991). The integrins are also likely to be involved the establishment of epithelial polarity in the kidney (Klein *et al*, 1988): function-inhibiting anti-laminin antibodies (preventing laminin-integrin interaction) prevent polarization in kidney tubules. Similarly, antibody inhibition of α_6 (which, as $\alpha_6\beta_1$, interacts with laminin) prevents kidney epithelial tubule development in organ culture (Sorokin *et al*, 1990). However, polarization is not necessary for β-casein synthesis in cultured mouse mammary gland epithelial cells, although milk protein synthesis is stopped if integrin function is blocked (Streuli *et al*, 1991).

Other than the naturally occurring *Drosophila* mutations, and inhibition of adhesion/migration with RGD peptides, integrin participation during morphogenesis has been correlative. There are numerous examples of changes in integrin expression patterns during development and differentiation. Dudley *et al* (Dudley *et al*, 1989) found an increase in the CD11c integrin subunit (alpha X; partners with β_2) both in TPA-induced differentiation of HL-60 cells to monocytes/macrophages *in vitro* and monocyte/macrophage differentiation *in vivo*. Murine erythroleukemia (MEL) cell attachment to a fibronectin matrix results in differentiation to mature erythrocytes, whereas in suspension differentiation is arrested. Both *in vitro* (in MEL) and *in vivo*, adhesion to fibronectin is down-regulated by fibronectin receptor ($\alpha_5\beta_1$) down-regulation late in erythrocyte development, permitting detachment of erythrocytes and exit from the bone marrow (Patel and Lodisch 1987). During kidney morphogenesis, integrin subunits are expressed in distinct kidney structures and the intensity of integrin staining also varies at different stages, suggesting integrin involvement (Korhonen *et al*, 1990a; Korhonen *et al*, 1990b). Integrins have been detected as early as the 4-cell stage of *Xenopus* development; although they are mostly in the immature form, they are found at cell-cell margins (Gawantka *et al*, 1992); this suggests that they may be involved in very early morphogenesis.

Integrins therefore have been demonstrated to participate in a limited number of morphogenetic processes involving cell-cell and cell-ECM interaction to date; there is additionally a large amount of

circumstantial evidence for integrin involvement in development and differentiation.

3.1.3 Approach

In the experiments described here, I have used immuno-fluorescence microscopy to document the integrins expressed in mature epidermis and at different stages of epidermal development. I have attempted to correlate the changes observed with changes in expression of three integrin ligands, fibronectin, type IV collagen and laminin, and of bullous pemphigoid antigen (BPA), in the basement membrane zone (BMZ). Finally, I have shown that the changes in integrin expression that occur at the onset of stratification *in vivo* can be reproduced in an organ culture model (Fisher and Holbrook 1987), a finding that offers significant potential for further studies of the role of integrins in assembly of the epidermis.

3.2 Results

Using immunofluorescence microscopy, the expression pattern of a number of integrin subunits was determined, from 7.8 weeks EGA to neonatal. 16 samples in the embryonic period (7.8 to 9.5 weeks EGA), 8 in the early fetal period (9.5 to 11 weeks), and 6 in the later fetal period (to 16.6 weeks) were analyzed in detail. Five foreskin samples were used; these represented neonatal, fully differentiated epidermis. The sample range covered the principal morphogenetic periods of the epidermis.

3.2.1 Integrin subunit expression *in vivo*

3.2.1.1 Neonatal epidermis

It was a prerequisite to determine the integrin subunits that were expressed in mature keratinocytes, from a large family of possible integrins. Although this had been examined by several investigators in cultured keratinocytes (Adams and Watt 1990; Carter *et al*, 1990; De Luca *et al*, 1990; Staquet *et al*, 1990; Adams and Watt 1991; Nicholson and Watt 1991), and to various degrees *in vivo* (Fradet *et al*, 1984; Sonnenberg *et al*, 1986; Wayner *et al*, 1988; De Strooper *et al*, 1989; Peltonen *et al*, 1989; De Luca *et al*, 1990; Adams and Watt 1991), there had not been a comprehensive *in vivo* survey. The distribution of integrin subunits in neonatal foreskin I detected is summarized in Table 1.

Expression of all integrins was largely confined to the basal layer, although faint staining of the first 1-2 suprabasal layers was sometimes seen (e.g. Fig. 1G) for the more strongly staining integrins. All basal cells expressed integrins and each of the subunits was expressed on all surfaces of the basal cells (i.e. pericellular staining), although staining appeared particularly strong between cells. Antibodies to α_v, β_1, and subunits known from immunoprecipitation studies of cultured keratinocytes to associate with β_1 (i.e. α_1, α_2, α_3 , α_5; (Adams and Watt 1990; Staquet *et al*, 1990; Adams and Watt 1991)) stained all basal cell surfaces uniformly, including the apical region. In contrast, α_6 and β_4 showed strongest expression at the basal surface of the basal cells, in contact with the basement membrane.

Table 1. Integrin expression in neonatal foreskin and in palm/sole skin during development.

Subunit	Expression in neonate	Expression during development
α_1	peribasal (faint)	high level of expression until ~15 weeks
α_2	peribasal	absent prior to stratification, or occasional patches of positive cells. Expressed at stratification and thereafter, especially strong in developing sweat glands
α_3	peribasal	expressed prior to stratification and throughout development
α_4	absent	absent
α_5	peribasal (faint)	not expressed until ~ 15 weeks; stronger in developing sweat glands
α_6	peribasal; conc. at basal surface	peribasal prior to stratification; thereafter concentrated at basal surface
α_v	peribasal (faint)	only expressed at stratification and thereafter, in about half of specimens; stronger in developing sweat glands.
β_1	peribasal	expressed prior to stratification and throughout development
β_3	absent	absent
β_4	peribasal; conc. at basal surface	as for α_6

Expression of β_4 appeared weaker than α_6 and was faint on the lateral and apical membranes (Fig. 7D).

Staining for the β_1, α_2, α_3 , α_6 and β_4 subunits (Figs. 1G, 3E, 4D, 6D, and 7D) was intense; in contrast, staining for α_1, α_5, and α_v (Figs. 2B, 5C, and 8C) was weak and virtually absent in some samples. α_4 and β_3 were never detected in the epidermis although blood vessel walls (Belkin *et al*, 1990; Defilippi *et al*, 1991b) and some cells within the blood vessels (Wayner *et al*, 1989) were stained, providing a positive control.

3.2.1.2 Embryonic and fetal stages

Having established the distribution of integrins in mature epidermis, I examined expression of the same integrins at three different stages of epidermal development: prior to stratification (embryonic period; up to 9.5 weeks EGA); immediately after stratification (early fetal; 9.5 to 11 weeks EGA, although stratification was sometimes observed earlier); and mid-development (11 to 17 weeks EGA). The results are summarized in Table 1.

Palm and sole skin were chosen for analysis because, particularly at the earliest stages of development, the morphology and integrity of the tissue was better than that from other body sites. In some specimens, arm or leg skin was also examined; the results obtained were essentially the same as for the palm and sole except that development of arm and leg epidermis was relatively less advanced than palm and sole at the same EGA (Holbrook and Odland 1980). Palm and sole skin lack hair follicles but contain abundant eccrine sweat glands, which start to develop at about 12 weeks EGA (Holbrook 1991).

3.2.1.2.1 Periderm

At all stages of development, the periderm did not express any of the integrins examined, with the possible exception of α_v, which showed occasional, speckled staining of individual cells, mainly in older specimens (not shown). In older specimens, the periderm was usually

(Text continues on p. 132)

Figure 1 β_1 integrin expression during development and in neonatal epidermis, with corresponding phase contrast micrographs showing morphology of key developmental stages. (A, B) 8.0 weeks EGA; (C, D) 9.9 weeks; (E, F) 13.6 weeks; and (G, H) neonatal skin.

Scale bar = 50 μm.

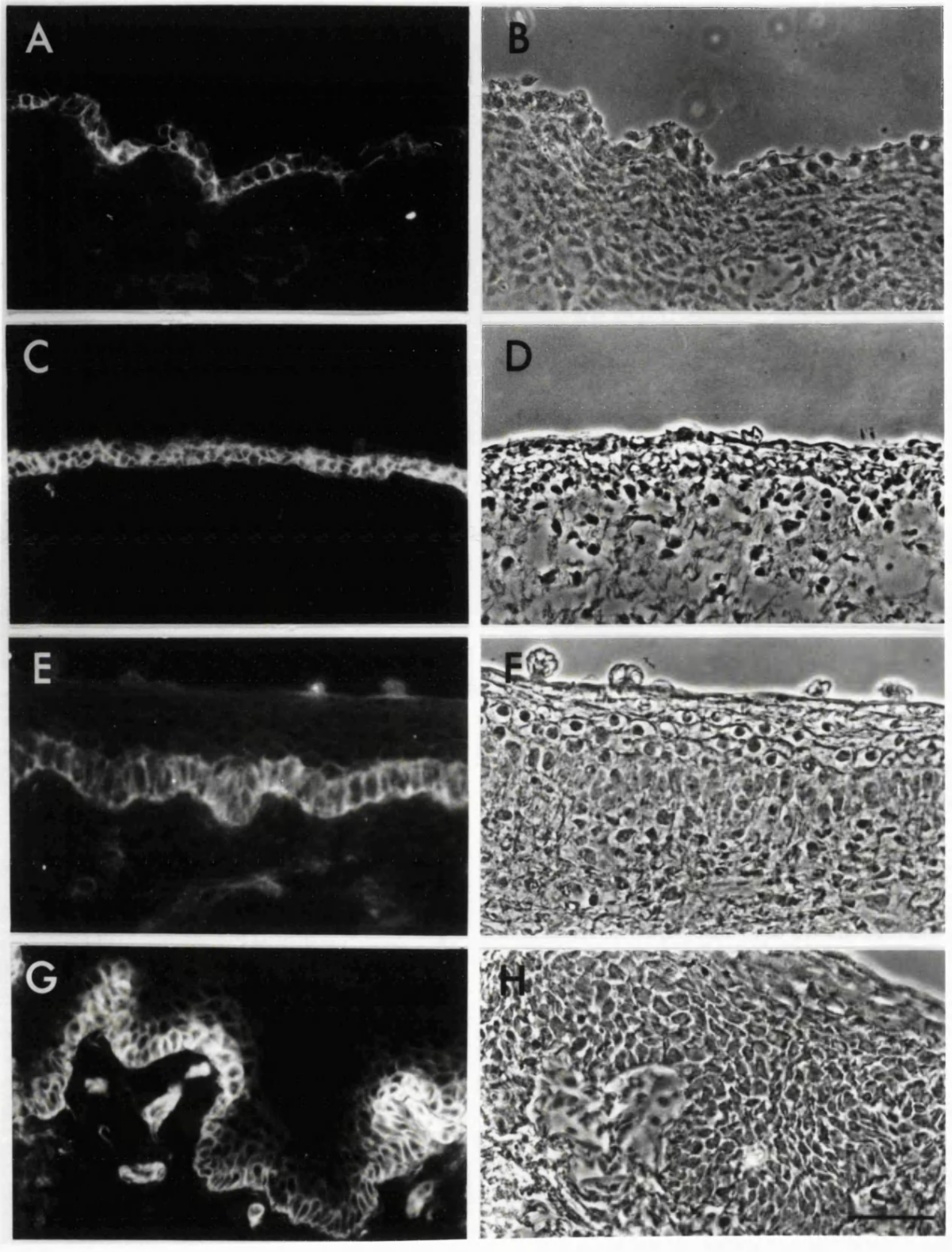

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Figure 2 α_1 integrin expression during development and in neonatal epidermis. (A) 9.0 weeks and (B) neonatal skin. Note that the sample in A had just stratified.

Scale bar = 50 μm.

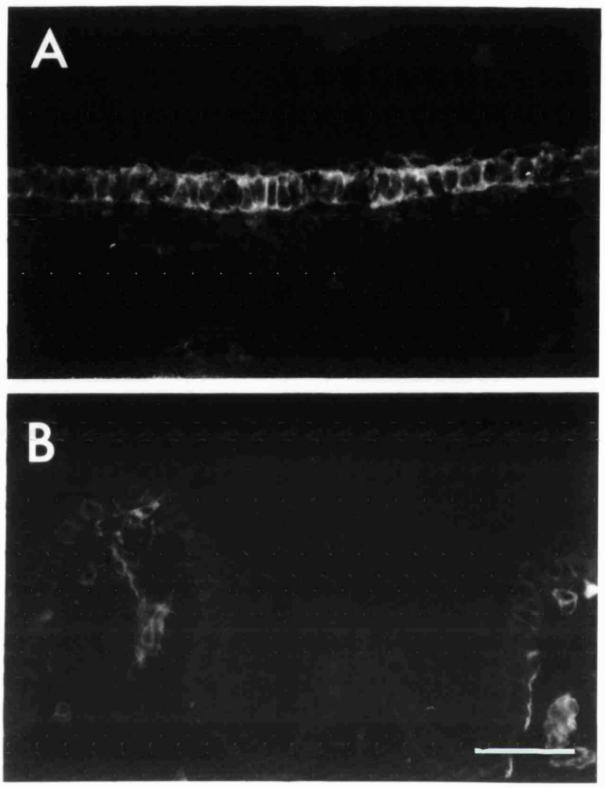
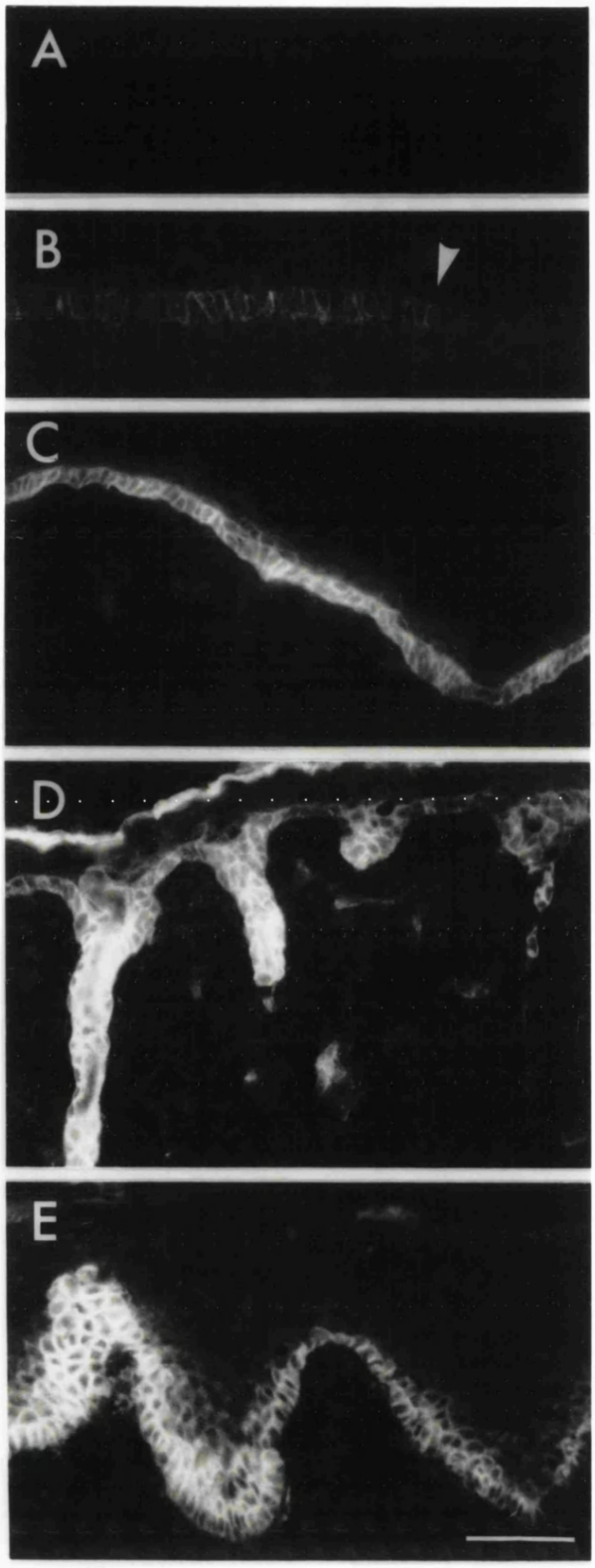

A
B

Figure 3 α_2 integrin expression during development and in neonatal epidermis. (A) 7.8 weeks, negative specimen; (B) 9.0 weeks, arrowhead indicates start of negative area to right; (C) 10.7 weeks, stratified; (D) 15.3 weeks, note intense sweat duct staining; and (E) neonatal skin.

Scale bar = 50 μm.


A
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E

Figure 4 α_3 integrin expression during development and in neonatal epidermis. (A) 8.0 weeks; (B) 10.7 weeks, stratified; (C) 13.6 weeks; and (D) neonatal foreskin.

Scale bar = 50 μm.

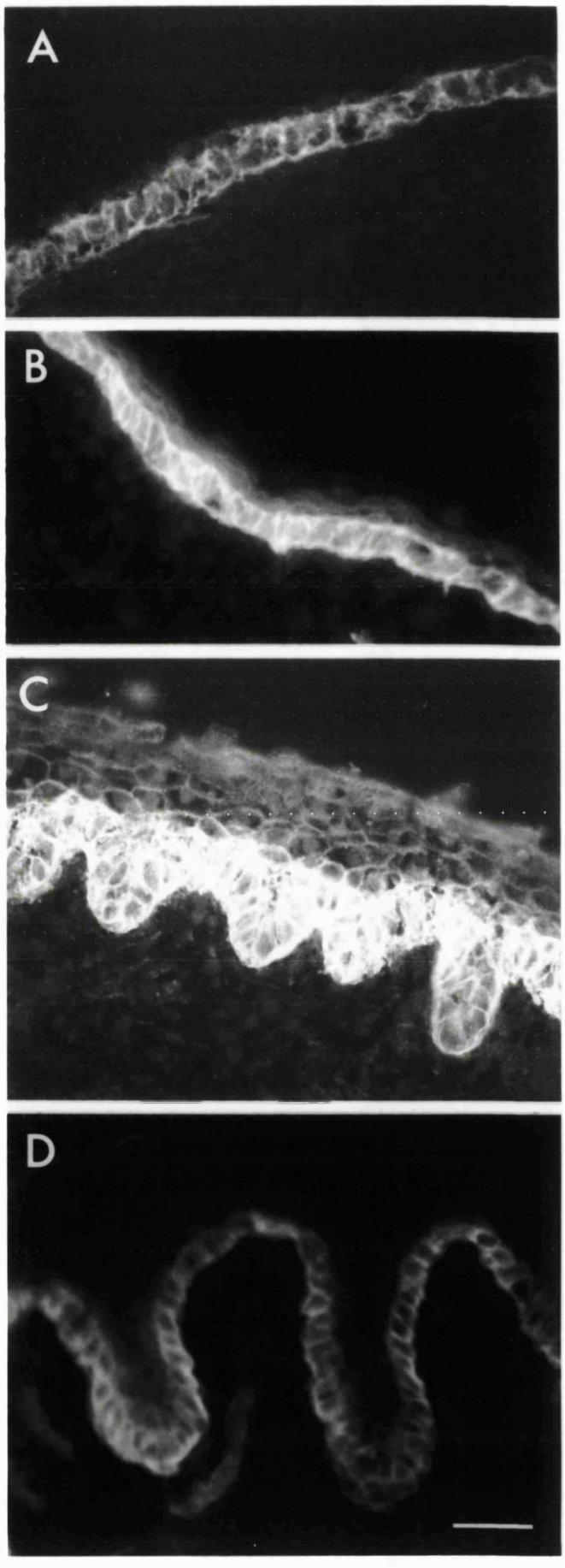
A
B
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Figure 5 α_5 integrin expression during development and in neonatal epidermis. (A) 8.0 weeks; (B) 15.3 weeks; arrow indicates elevated expression in developing sweat gland; and (C) neonatal skin.

Scale bar = 50 μm.

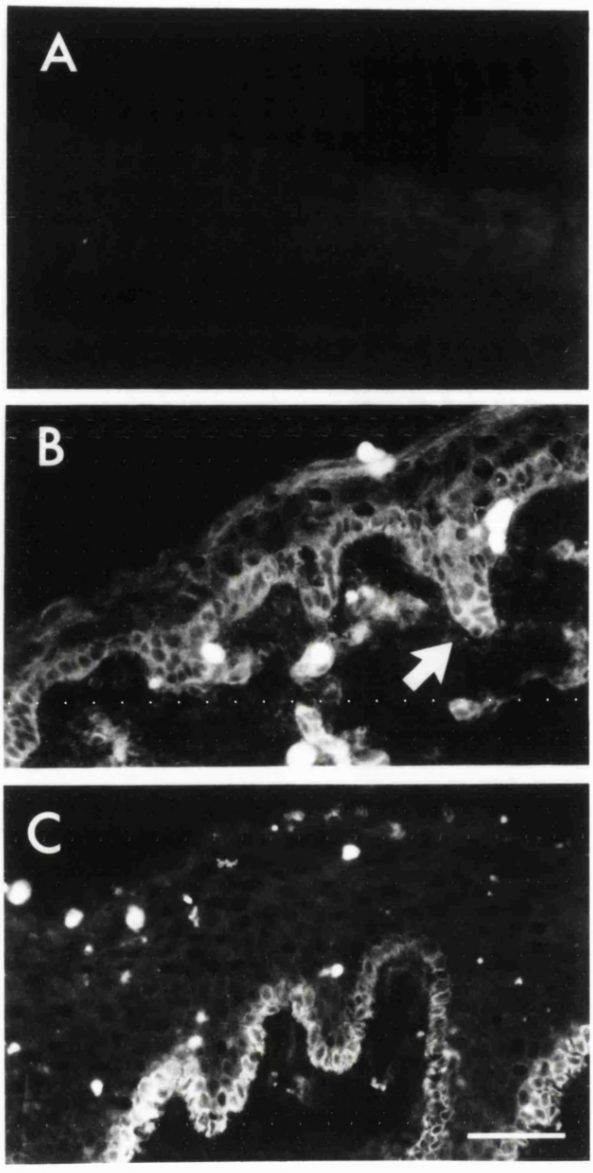
A
B
C

Figure 6 α_6 integrin expression during development and in neonatal epidermis. (A) 8.6 weeks; (B) 9.0 weeks; (C) 13.6 weeks; and (D) neonatal skin. Note that the sample in B had just stratified.

Scale bar = 50 μm.

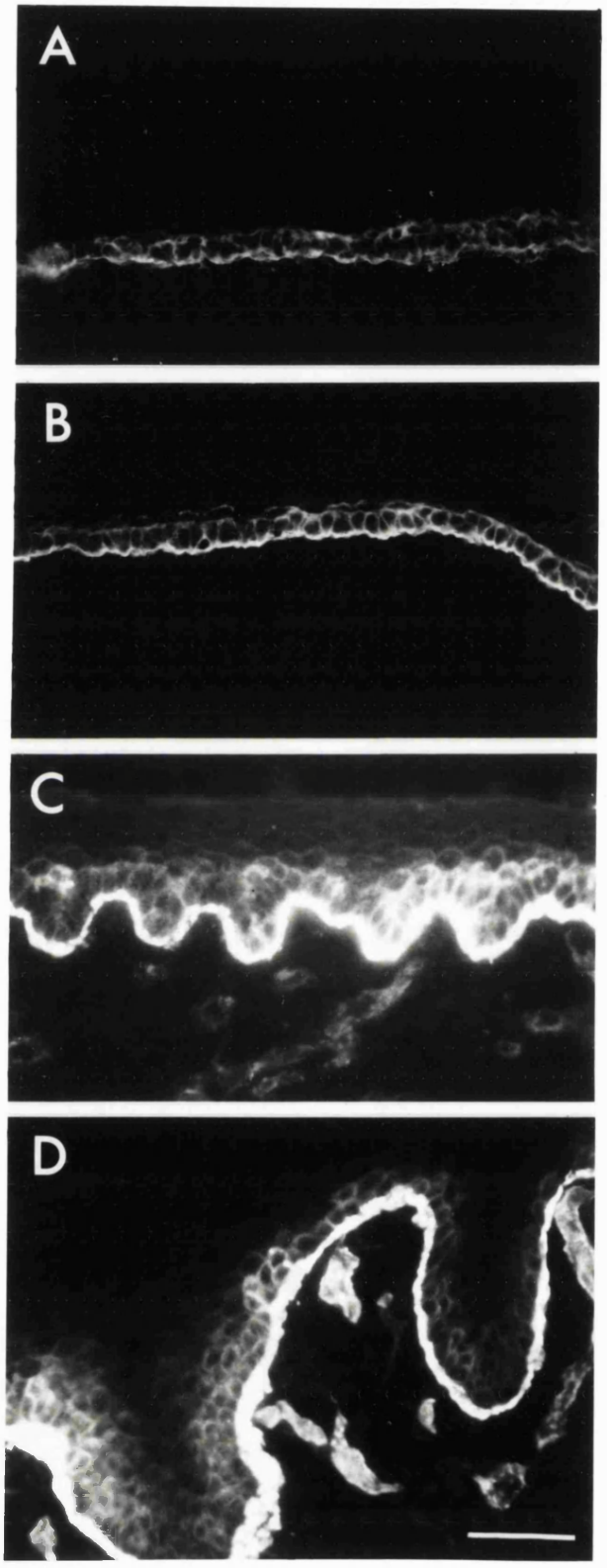
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Figure 7 β_4 integrin expression during development and in neonatal epidermis. (A) 7.8 weeks; (B) 10.7 weeks, stratified; (C) 13.6 weeks; and (D) neonatal foreskin.

Scale bar = 50 μm.

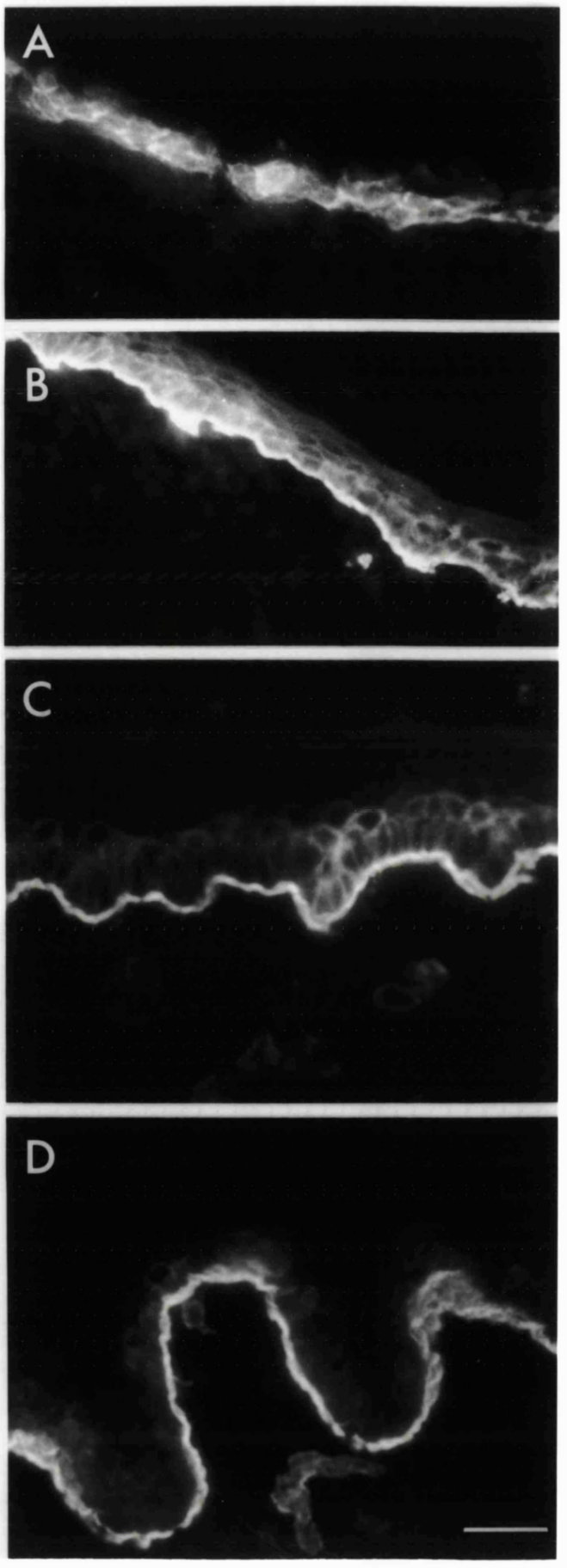
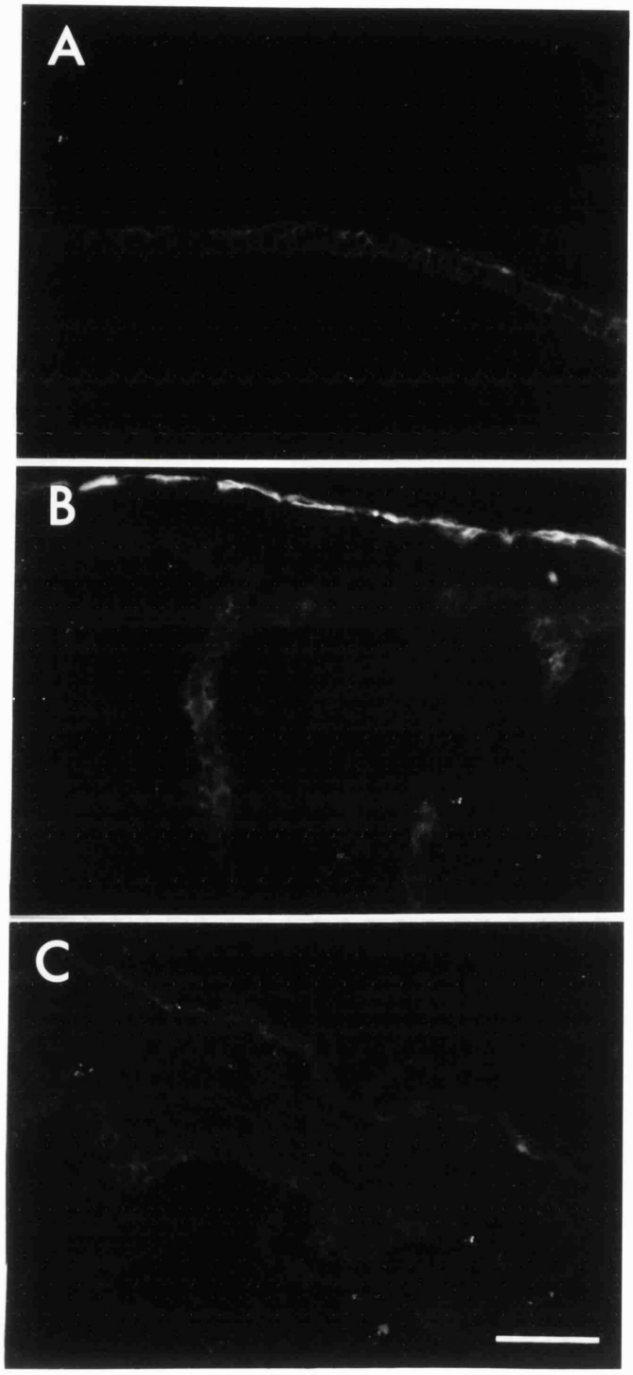

A
B
C
D

Figure 8 α_v integrin expression during development and in neonatal epidermis. (A) 9.0 weeks; (B) 15.3 weeks, the periderm staining is non-specific; and (C) neonatal skin. Note that the sample in A had just stratified.

Scale bar = 50 μm.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B
C

Figure 9 E-cadherin expression during development and in neonatal epidermis. (A) 9.4 weeks, note that the lateral, but not apical, membranes of the periderm appear stained; (B) 10.7 weeks; and (C) neonatal foreskin.

Scale bar = 50 μm.

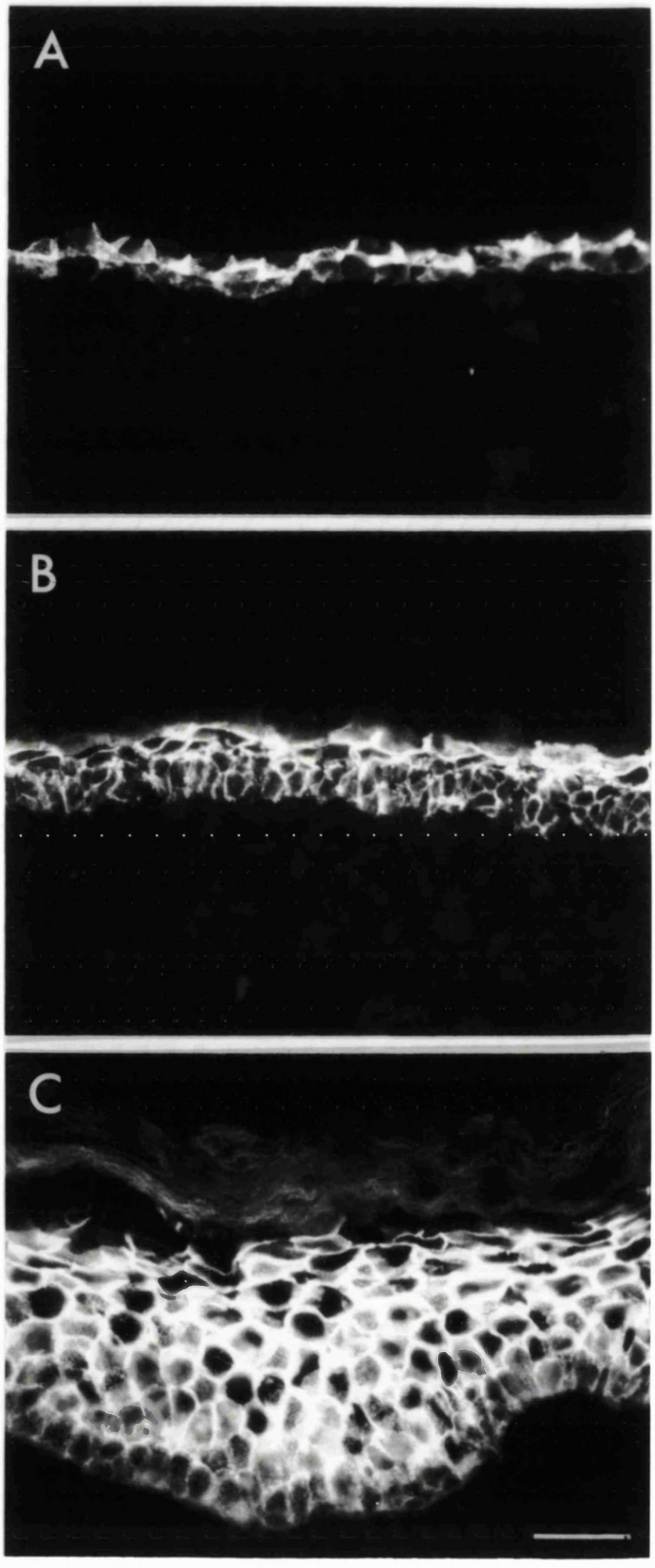
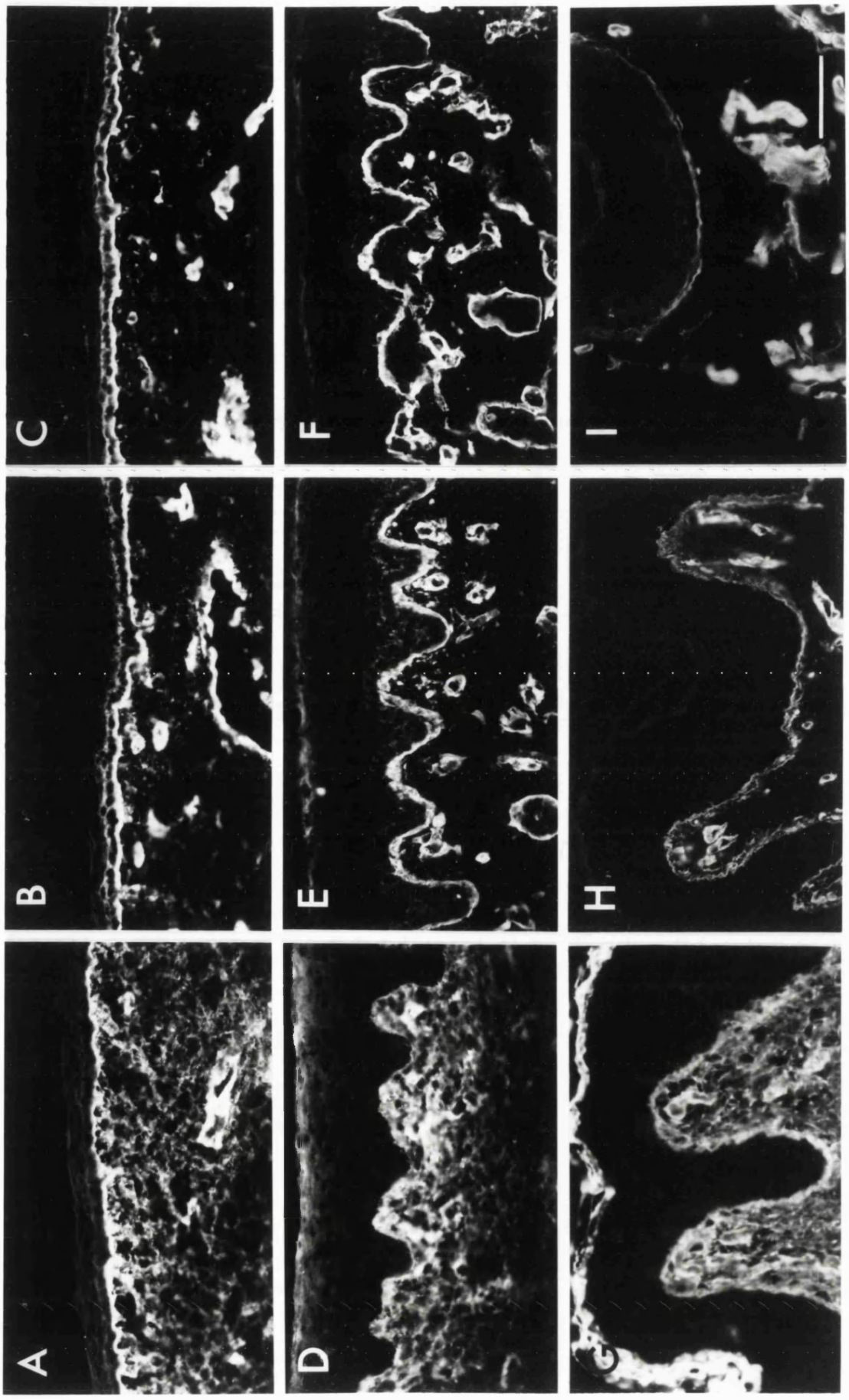

A
B
C

Figure 10 ECM protein localization at three key stages. (A-C) 10.7 weeks, just after stratification; (D-F) 13.6 weeks; and (G-I) neonatal skin. (A, D, G) fibronectin; (B, E, H) collagen type IV; (C, F, I) laminin. The basement membrane zone can be identified by the linear staining separating the dermis and epidermis, in for example, E.

Scale bar = 50 μm.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stained by all antibodies (e.g. Fig. 3D), though this was probably non-specific because controls using second antibody alone also stained the periderm.

3.2.1.2.2 Prestratification (embryonic period)

At this early stage in development, the cells already expressed several of the integrin subunits that were present in mature epidermis: β_1, α_1, α_3 , α_6, and β_4 (Figs. 1A, 4A, 6A, 7A, and Table 1). These integrins had a uniform pericellular distribution; for α_6 and β_4, this was in contrast to the concentration at the BMZ noted in neonatal skin (compare Fig. 6A with D; and 7A with D). Staining for α_1 was more intense in embryonic epidermis than in foreskin (results not shown but comparable to Fig. 2A).

Two integrins — α_5 and α_v — detected in mature epidermis were not observed at this stage of development. α_5 was never detected prior to stratification (Fig. 5A) and α_v was detected in only one out of 16 specimens (not shown). To eliminate the possibility of problems with a particular antibody, the staining pattern using two anti-α_v monoclonal antibodies was compared and found to be identical (not shown). The α_4 and β_3 subunits, which were not present in mature epidermis, were also absent in embryonic epidermis.

α_2 was not expressed in the youngest specimens examined (n=3 at 7.8 weeks EGA; see Fig. 3A). However, α_2 showed patchy expression in about half of the other prestratification specimens. When present, α_2 expression was pericellular and confined to stretches of faintly positive cells bordered by negative areas (Fig. 3B). There was no apparent difference in epidermal morphology between the positive and negative areas. The same staining pattern was observed with three different anti-α_2 monoclonal antibodies.

3.2.1.2.3 Onset of stratification (fetal period)

The fetal stage of development starts at the onset of stratification, when an intermediate layer of keratinocytes forms between the basal and

periderm layers at about 9.5 weeks EGA. This is one of the key morphogenetic events in the epidermis, representing the first appearance of terminally differentiating keratinocytes.

The β_1, α_1, and α_3 integrin subunits continued to be uniformly and strongly expressed in a pericellular distribution in the basal layer of newly stratified epidermis (Figs. 1C, 2A, and 4B). The α_4, α_5 (Fig. 5A), and β_3 subunits were not detected, whereas α_v was very faintly expressed in the basal layer in 5 out of 8 specimens examined (Fig. 8A). α_6 and β_4 were expressed in the basal layer of keratinocytes, but in contrast to the prestratification distribution (Fig. 6A, 7A), there was a concentration of staining at the basal surface of the basal cells (Fig. 6B, 7B), as observed in neonatal epidermis (Fig. 6D, 7D). Whereas prior to stratification, α_2-positive cells were found in patches (Fig. 3B), after stratification α_2 was expressed by all basal keratinocytes in 7 out of 8 specimens examined (Fig. 3C). In the other specimen, no positive staining was observed, even though the specimen showed strong expression of the other integrins.

3.2.1.2.4 Further development

Between 12 and 17 weeks of gestation more layers of differentiated keratinocytes are added, reaching a total of 5-6 by 17 weeks. In the oldest specimen examined, cornified layers were beginning to form (e.g. Fig. 3D). As observed at the earlier stages of development, integrin expression was largely confined to the basal layer. The β_1 and α_3 subunits continued to show strong pericellular expression (Figs. 1E and 4C). α_1 was also expressed, but starting from about 15 weeks of gestation onwards the intensity of staining started to decrease to the low level observed in neonatal skin (Fig. 2B). α_6 and β_4 staining was concentrated at the BMZ, but there was also clear pericellular staining of the basal cells and faint staining of the first suprabasal layers (Fig. 6C and 7C).

Three of the integrin subunits, α_2, α_5, and α_v, appeared to be expressed at a higher level in developing sweat glands than in the regions between the sweat glands. α_2 was expressed in all specimens of epidermis (Fig. 3D), but α_5 was detected in about half of the specimens and then at a very low level (Fig. 5B), as previously reported (Peltonen *et al*, 1989);

although Wayner *et al* (Wayner *et al*, 1988) observed strong staining in all keratinocyte layers. Staining with α_v was also faint, comparable to α_5, and was only detected in about half of the samples (Fig. 8B).

3.2.2 E-Cadherin

The expression pattern of E-cadherin, a calcium-dependent cell-cell adhesion molecule which has been suggested to be central to epidermal morphogenesis (Wheelock and Jensen 1992), was also examined. Expression in all keratinocyte layers could be detected in foreskin (Fig. 9C), as reported previously (Hirai *et al*, 1989; Shimoyama *et al*, 1989; Wheelock and Jensen 1992). Similarly, E-cadherin could be detected in pre-stratification keratinocytes as well as periderm cells (Fig. 9A), except at the periderm apical margin (Fujita *et al*, 1992). In older specimens, some periderm cells appeared negative (Fig. 9B). In all specimens, the BMZ side of the basal cells appeared negative.

3.2.3 Basement membrane components

The expression of fibronectin, collagen type IV, and laminin — potential integrin ligands present in epidermal basement membranes — was examined to identify any changes in their distribution during development. Fibronectin was detected at high levels in the dermis throughout development. Staining formed a bright line at the BMZ early in development (Fig. 10A), until about 13 weeks EGA, becoming less apparent thereafter (see Fig. 10D, G; see also (Fine *et al*, 1984)). The dermis was strongly positive at all times. Collagen type IV and laminin staining were concentrated in the BMZ and around blood vessels throughout development, with no apparent changes in levels of expression during development (Fig. 10 B-I), as reported previously (Fine *et al*, 1984). Just after stratification, faint staining of collagen type IV and laminin was also observed along the lateral and apical surfaces of the basal cells (Fig. 10B, C).

3.2.4 Organ culture — modeling epidermal development *in vitro*

3.2.4.1 *In vitro* integrin expression at stratification

Staining with antibodies to integrin subunits showed that major changes in integrin expression occur at the onset of stratification. It has previously been reported that embryonic skin maintained in organ culture can undergo near-normal development, although at an accelerated rate (Fisher and Holbrook 1987). I therefore investigated whether such cultures would provide an experimental model for analyzing the significance of changes in integrin expression observed *in vivo*.

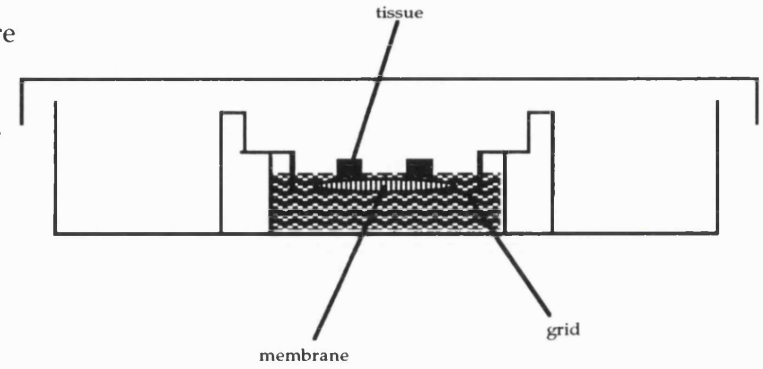


Figure 11. Skin organ culture model. Tissue is shown on top of Millipore membrane, supported by a grid. Medium almost submerges the tissue.

Prestratification specimens of palm or sole tissue were dissected into several pieces. One piece was frozen immediately and the others were cultured for 15-24 hours, then frozen. The histological appearance of cultured epidermis is shown in Fig. 12B, D and F. In every case, stratification had taken place in culture, and the cell layers were almost indistinguishable from layers formed after stratification *in vivo* (compare with Fig. 1D). A total of 7 cultures was studied.

Cultures were stained with antibodies to α_2 and α_6, two subunits that underwent marked changes on stratification, and with antibodies to β_1 and α_3. α_2 expression was absent or patchy prior to stratification but, after culture, I detected uniform expression in the basal layer (Fig. 12C). α_6 showed uniform pericellular distribution prior to stratification but, after culture, there was a concentration of stain at the BMZ (Fig. 12E). β_1 (Fig.

Figure 12 Integrin redistribution in organ cultured pre-stratification skin. (A) β_1, 8.3 week specimen after 22-hour culture, with (B) corresponding phase micrograph; (C) α_2, 8.3 week specimen after 22-hour culture, with (D) corresponding phase micrograph; (E) α_6, 8.6 week specimen after 15-hour culture, with (F) corresponding phase micrograph; (G,H) dual labeling of 8.6 week specimen after 24-hour culture: (G) α_6; (H) BPA on same field. On single-labeled specimens, there was fluorescence only with the appropriate filter.

Scale bar = 50 μm for A-F, scale bar = 12.5 μM for G and H.

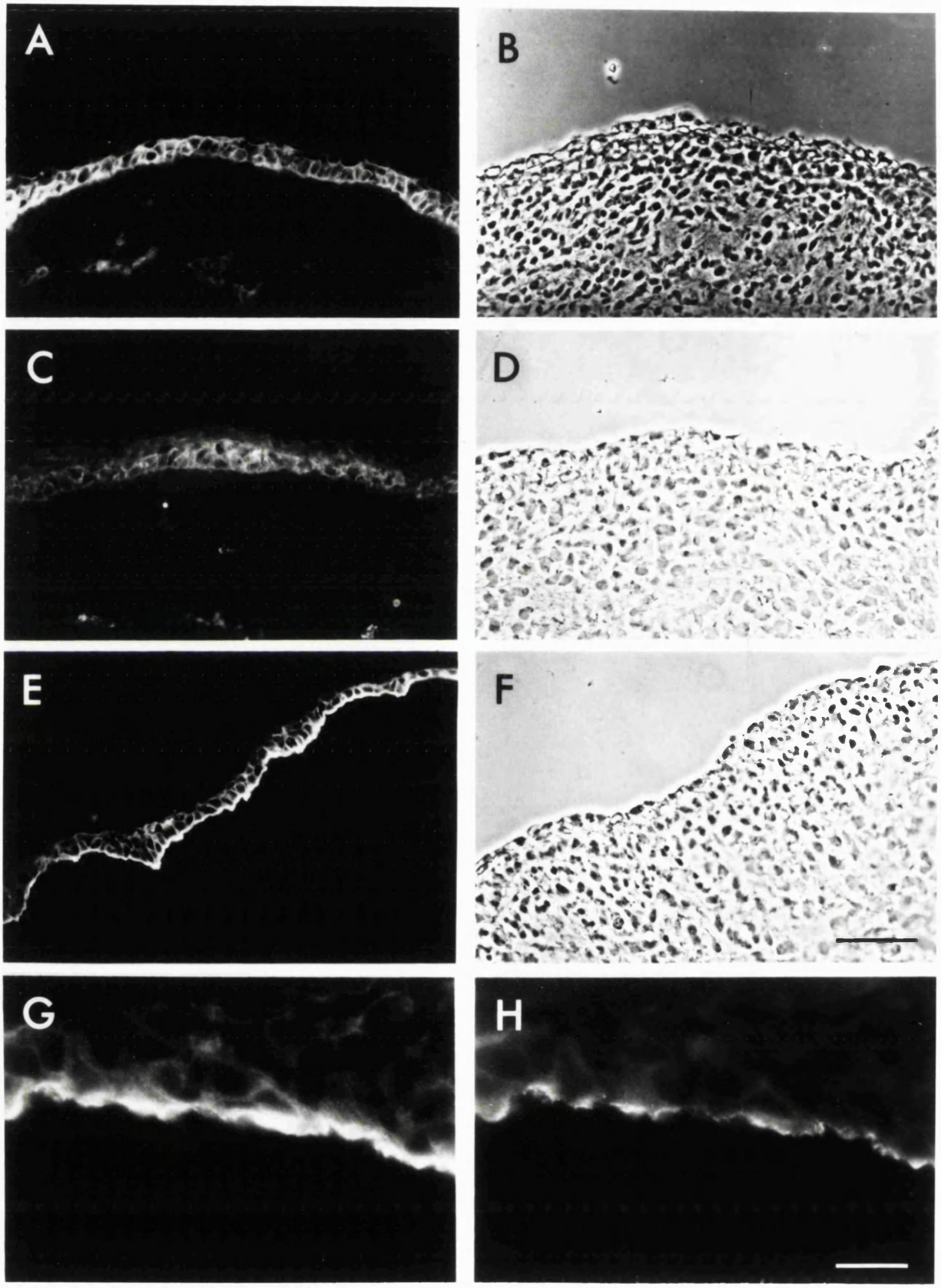
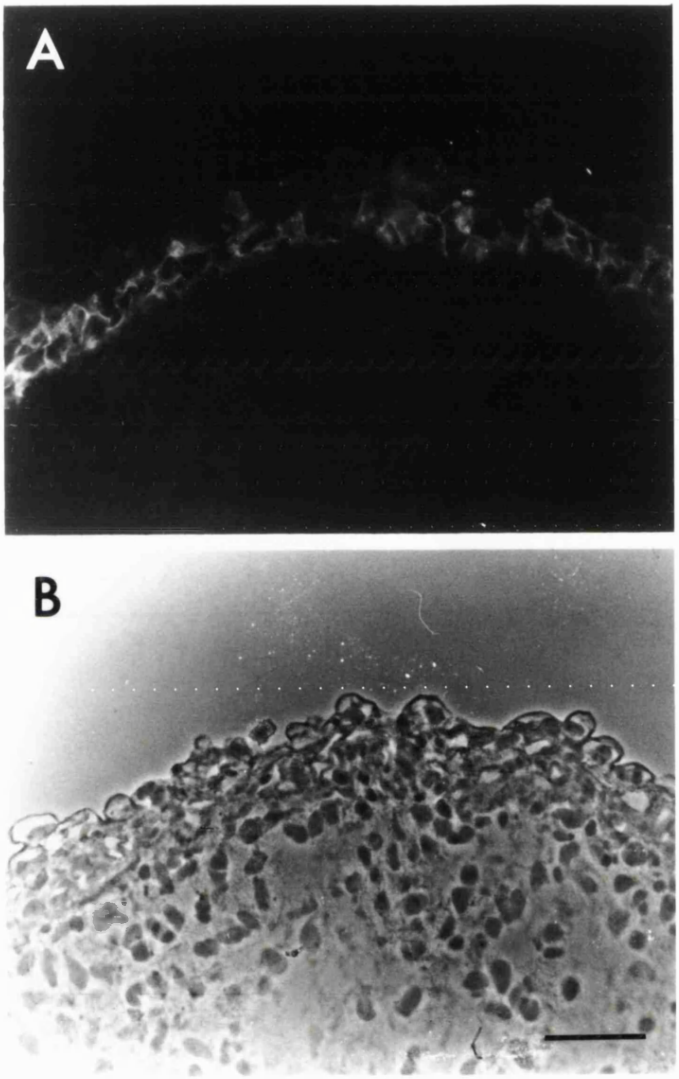

A
B
C
D
E
F
G
H

Figure 13 Labeling of basal keratinocytes (A) and morphology (B) after overnight culture of pre-stratification skin in anti-α_2 integrin antibody.

Scale bar = 50 μm.


A
B

12A) and α_3 (not shown) continued to be expressed in the basal layer following stratification. Thus the patterns of integrin staining *in vitro* were identical to those seen *in vivo*.

α_6 and β_4 localize to hemidesmosomes at the BMZ (Carter *et al*, 1990; Kurpakus *et al*, 1990; Stepp *et al*, 1990; Kurpakus *et al*, 1991; Sonnenberg *et al*, 1991; Fine *et al*, 1992). Hemidesmosomes are first formed at the onset of stratification in the epidermis (Fine *et al*, 1984; Lane *et al*, 1985). I therefore examined whether the redistribution of α_6 to the BMZ correlated with the appearance of hemidesmosomes, as determined by staining for bullous pemphigoid antigen (BPA). Staining for BPA was rarely present at the BMZ before stratification (not shown), but appeared discontinously at the BMZ following culture, consistent with the appearance of BPA following stratification *in vivo* (Fig. 12H). Dual labeling of stratified epidermis with BPA and α_6 antibodies showed that BPA co-localized with α_6 at the BMZ (Fig. 12G and H), but, in contrast to BPA, α_6 staining was uniform along the BMZ and pericellular, as described above.

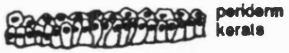
3.2.4.2 Antibody penetration of organ cultures

One potential application of embryonic skin organ culture is to analyze the role of the integrins in stratification by attempting to perturb the process with anti-integrin antibodies. The appearance of α_2 showed a correlation with stratification, suggesting that it may be crucial for stratification to occur. α_2 has also been implicated in cell-cell adhesion, a vital requirement for a stratified cell.

As a preliminary experiment, I cultured embryonic (unstratified) epidermis overnight in the presence of 200μg/ml monoclonal anti-α_2 (HAS-6). Antibody was able to penetrate the tissue and could be localized in the basal cells (Fig. 13). Although stratification still occurred, it is not possible to eliminate a role for α_2 as the antibody I used is not a fully function-blocking antibody (Tenchini *et al*, 1993); repeating the experiment with a better function-blocking antibody is necessary.

Figure 14. Diagram illustrating the stages of epidermal development examined and the main changes in integrin expression at each stage. The different layers of neonatal epidermis are indicated. Intermed. = intermediate, kerats. = keratinocytes.

8 weeks
embryonic



α2 absent or patchy

α6, β4:uniform pericellular staining

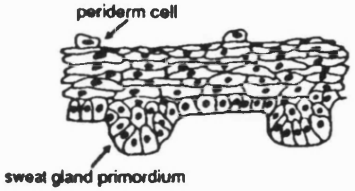
10 weeks
fetal

α2 expressed by all basal keratinocytes

α6, β4 concentrated at BMZ

αv first expressed

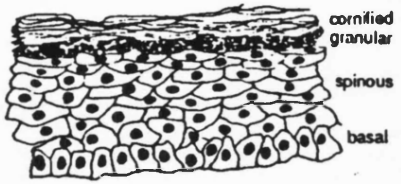
14 weeks



α1 declines after this stage

onset of expression of α5

neonate



3.3 Discussion

Integrin-mediated interactions between cells and extracellular matrix molecules play an important role in differentiation and morphogenesis (see, for example: (Menko and Boettiger 1987; Patel and Lodisch 1987; Korhonen *et al*, 1990a; Sorokin *et al*, 1990). In the epidermis, integrins are involved not only in maintaining the spatial organization of keratinocytes but also in regulating the initiation of terminal differentiation (Adams and Watt 1989; Adams and Watt 1990; Hotchin and Watt 1992). I have begun to investigate the role of integrins in the development of the epidermis from a single layer of keratinocytes to the mature stratified tissue, i.e. the establishment of epidermal structure.

I have examined the pattern of integrin expression in human epidermis at different stages of gestation, and compared it with the distribution of receptor ligands within the basement membrane. I have also determined that the changes observed during development *in vivo* can be reproduced in organ culture, thus providing the opportunity to analyze factors that regulate integrin expression and initiation of stratification. Wherever possible, I have used more than one antibody to each integrin subunit, using a biotin-streptavidin detection system to maximize sensitivity. The different stages of epidermal development that I examined and the major changes in integrin expression I observed are summarized in Figure 14.

The staining that I found in neonatal (i.e. mature) epidermis largely confirms the observations of others (Kajiji *et al*, 1987; De Strooper *et al*, 1989; Peltonen *et al*, 1989; Carter *et al*, 1990; Klein *et al*, 1990; Nazzaro *et al*, 1990). Integrin expression was mainly confined to the basal layer and there was no evidence of basal cell heterogeneity. All of the integrins showed a pericellular distribution, although α_6 and β_4 were concentrated at the BMZ. The strongest staining detected was for integrin subunits α_2, α_3, β_1, α_6, and β_4. I also detected very weak staining for α_1, α_5, and α_v; there is some controversy about the presence of these subunits in the epidermis and this is discussed for each subunit below. These results are in agreement with data on the relative abundance of different integrins obtained from immunoprecipitation of metabolically labeled cultured keratinocytes (De Luca *et al*, 1990; Larjava *et al*, 1990; Adams and Watt

1991). It is not possible to determine which integrin subunits form heterodimers on the basis of immunofluorescence staining, but the immunoprecipit-ation studies show that α_2, α_3 and α_5 are complexed with β_1, α_6 and β_4 form a heterodimer and α_v is probably expressed in association with β_5 (De Luca *et al*, 1990; Staquet *et al*, 1990; Adams and Watt 1991).

The integrin subunits expressed in developing epidermis were the same as those expressed in the neonatal, mature tissue. As in mature epidermis, the integrins were largely confined to the basal layer. However, with the exception of $\alpha_3\beta_1$, all of the integrins showed temporal or spatial variation in expression.

3.3.1 $\alpha_1\beta_1$

$\alpha_1\beta_1$ was expressed prior to epidermal stratification and staining was relatively strong until about 15 weeks EGA. Later in development and in neonatal epidermis I observed only weak staining; others have reported it to be present in trace amounts (Belkin *et al*, 1990; Buck *et al*, 1990) or absent in mature epidermis (Hemler *et al*, 1984; De Luca *et al*, 1990; Nazzaro *et al*, 1990). Using immunoprecipitation, $\alpha_1\beta_1$ is not detected consistently in cultured keratinocytes, but it is expressed by ndk, a strain of epidermal cells with a complete block in terminal differentiation (Adams *et al*, 1991). This integrin has been shown to bind to collagen types I-IV (Kramer and Marks 1989; Belkin *et al*, 1990; Defilippi *et al*, 1991b), and to the E1 region of laminin (Ignatius and Reichardt 1988; Hall *et al*, 1990). The ligand for the keratinocyte $\alpha_1\beta_1$ has yet to be determined, but until about 15 weeks EGA, $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ are all highly expressed; it is therefore likely that the basal keratinocytes are simultaneously expressing two laminin or collagen receptors. An intriguing possibility is that different receptors for the same ECM ligand are coupled to different second messenger pathways or cytoskeletal components.

There are a number of possible explanations for the decrease in $\alpha_1\beta_1$ staining during epidermal development. A formal possibility, which could also apply for the other subunits, is that α_1 is present at the same level, but the epitope recognized by the antibody becomes masked; this can

only be tested by extraction and biochemical analysis. A second possibility is that decreased $\alpha_1\beta_1$ expression reflects changes in basement membrane composition. Laminin and collagen type IV have recently been shown to be products of multigene families and different isoforms are present in basement membranes of different tissues (Sanes *et al*, 1990; Horwitz 1991; Sollberg *et al*, 1992); other modifications such as laminin glycosylation could also play a role in $\alpha_1\beta_1$ affinity and expression (Chammas *et al*, 1991). The antibodies that I used might not detect such changes. Furthermore, the relative abundance of other collagen types (in particular, types III and V) at the dermo-epidermal junction changes during skin development (Smith *et al*, 1986), although there is, so far, no evidence for collagen type-specificity in integrin binding. It is intriguing that the reduction in α_1 staining was concurrent with the appearance of α_5 and decreased fibronectin staining at the BMZ; at present, there is no indication that this correlation is significant. Finally, it is interesting that in another tissue, the aorta, the level of $\alpha_1\beta_1$ also decreases with increasing gestational age (Belkin *et al*, 1990); down-regulation of α_1 may turn out to be a common phenomenon during development.

3.3.2 $\alpha_2\beta_1$

This has been reported to be a receptor for collagen types I-IV (Santoro 1988; Wayner *et al*, 1988; Kramer and Marks 1989; Staatz *et al*, 1989), laminin (Languino *et al*, 1989), and possibly fibronectin (Kirchhofer *et al*, 1990). In cultured keratinocytes, it is a collagen (Wayner *et al*, 1988; Carter *et al*, 1990; Staquet *et al*, 1990; Adams and Watt 1991) and laminin receptor (Carter *et al*, 1990). There is some evidence that $\alpha_2\beta_1$ may also play a role in cell-cell adhesion (Carter *et al*, 1990; Larjava *et al*, 1990; Lampugnani *et al*, 1991; Marchisio *et al*, 1991; Symington and Carter 1992); the nature of the intercellular ligand(s) is unknown, but it is interesting that at least for a short period of development (Fig. 10B and C), and also in cultured keratinocytes (Nicholson and Watt, unpublished data) and endothelial cells (Lampugnani *et al*, 1991), pericellular staining for laminin and collagen type IV is observed. Expression at stratification, concurrent with perhaps a greater need for cell-cell adhesion, is attractive in that adhesion of suprabasal cells (at their basal surface) to the basal cells (at their suprabasal

surface) may involve integrins. However, it is more likely that cadherin and desmosomes are the primary cell-cell adhesion molecules, as they are expressed by all suprabasal cells, and integrins are not.

$\alpha_2\beta_1$ was not expressed in the earliest specimens examined; in older pre-stratification specimens, it was sometimes present, but then only in a patchy distribution. From stratification onwards, α_2 was strongly stained in all cells of the basal layer, with particularly high expression in developing sweat ducts. α_2 was the only integrin subunit to show heterogeneous expression among basal cells and at present we can only speculate as to its significance: it could represent local variation in the composition of the basement membrane; or it might be a marker of areas that are about to stratify. Zutter and Santoro (Zutter and Santoro 1990) have noted that $\alpha_2\beta_1$ is highly expressed in proliferating populations of epithelial cells; another explanation for the patchy distribution prior to stratification is that it may reflect proliferative heterogeneity within the basal layer. The organ cultures that I have employed will be useful for testing these ideas.

3.3.3 $\alpha_3\beta_1$

This has been reported to be a collagen, laminin and fibronectin receptor (Wayner and Carter 1987; Takada *et al*, 1988; Gehlsen *et al*, 1989), using both RGD-independent and dependent mechanisms (Elices *et al*, 1991). The laminin binding site has been localized to the globular domain of the long arm (Gehlsen *et al*, 1989). In cultured keratinocytes, it acts as a laminin (Carter *et al*, 1990; Staquet *et al*, 1990; Adams and Watt 1991) and epiligrin (Carter *et al*, 1991; Rousselle *et al*, 1991) receptor. It is also found at cell-cell contacts in a variety of cells (Kaufmann *et al*, 1989) and may play a role in keratinocyte-keratinocyte adhesion (Carter *et al*, 1990; Larjava *et al*, 1990; Symington and Carter 1992). $\alpha_3\beta_1$ was the only integrin that showed no change in abundance or distribution in the epidermis; it was present in the basal layer prior to stratification and throughout development. Since it can bind to a range of ligands, it may fulfil multiple functions in the developing tissue and be present early as a cardinal integrin.

3.3.4 $\alpha_5\beta_1$

In keratinocytes and other cell types, the only reported ligand for $\alpha_5\beta_1$ is the central cell binding domain of fibronectin (Hynes 1987; Wayner *et al*, 1989; Adams and Watt 1990). Some cell types adhere to the IIICS site of fibronectin via $\alpha_4\beta_1$ (Wayner *et al*, 1989). However, keratinocytes do not adhere to IIICS (Adams and Watt 1990) and did not express α_4 in mature epidermis or at any stage of development.

α_5 was the last integrin subunit to be expressed during development. Expression was always low, although higher in the cells of developing sweat ducts than in the basal layer, as previously reported by Peltonen *et al* (Peltonen *et al*, 1989). The low level of $\alpha_5\beta_1$ expression is consistent with the low level of fibronectin in mature basement membrane (Stenman and Vaheri 1978; Fleischmajer and Timpl 1984) and the low (Staquet *et al*, 1990) to absent (Takashima and Grinnell 1985; Buck *et al*, 1990) $\alpha_5\beta_1$ expression reported for mature keratinocytes *in vivo*. It is, however, surprising that $\alpha_5\beta_1$ only appeared in the epidermis after fibronectin was no longer concentrated at the basement membrane zone. Tenascin is enriched in the dermis adjacent to the BMZ early in development, especially at sites of mesenchymal-epithelial interaction (Chiquet-Ehrismann 1991), e.g. sites of invasive sweat gland primordia. Since tenascin inhibits integrin binding to fibronectin (Chiquet-Ehrismann *et al*, 1988) it is conceivable that $\alpha_5\beta_1$, if present, would not bind fibronectin anyway. Our finding is similar to the observation of Korhonen *et al* (Korhonen *et al*, 1990a) that $\alpha_5\beta_1$ does not consistently codistribute with fibronectin in developing kidney. It is interesting to note that the expression of *Notch*, a determination gene originally described in *Drosophila*, is expressed in hair follicles as they interact with the underlying mesenchyme (Weinmaster *et al*, 1991), in a pattern reminiscent of α_2, α_5, and α_v expression.

Fibronectin binding is upregulated when keratinocytes are placed in culture (Toda *et al*, 1987; Grinnell 1992), yet although cultured keratinocytes adhere better to fibronectin than laminin or collagen type IV (Clarke *et al*, 1985; Adams and Watt 1990), immunoprecipitation of metabolically labeled cell extracts shows that $\alpha_5\beta_1$ is less abundant than $\alpha_2\beta_1$ and $\alpha_3\beta_1$ (Adams and Watt 1991). Further evidence that there need

not be a simple correlation between $\alpha_5\beta_1$ expression and function comes from the observation that during keratinocyte terminal differentiation, the ability of $\alpha_5\beta_1$ to bind fibronectin decreases prior to a decrease in the level of $\alpha_5\beta_1$ on the cell surface (Adams and Watt 1990).

In contrast to our findings and those of Peltonen *et al* (Peltonen *et al*, 1989), Wayner *et al* (Wayner *et al*, 1988) found that α_5 was present in all the epidermal layers of midgestation skin. One explanation for the discrepancy may be that the antibodies used recognize different epitopes; an abstract by De Strooper *et al* (De Strooper *et al*, 1989) reports that a monoclonal antibody to α_5 stains suprabasal but not basal keratinocytes in mature epidermis. At least in culture, the immunofluorescence localization of α_5 to the basal layer is supported by the finding that the levels of the protein detected by immunoprecipitation (Adams and Watt 1990) and of the α_5 mRNA (Nicholson and Watt 1991; Hotchin and Watt 1992) decrease during terminal differentiation.

3.3.5 α_V

This subunit can form heterodimers with β_1, β_3, or β_5 and can participate in binding to vitronectin, fibronectin, fibrinogen, von Willebrand factor, osteonectin, or thrombospondin (Bodary *et al*, 1989; Cheresh *et al*, 1989; Bodary and McLean 1990; Hemler 1990; Ramaswamy and Hemler 1990). Vitronectin has been reported to be absent from basement membrane (Reilly and Nash 1988); however, if α_V were complexed with β_1, it could be binding to fibronectin (Vogel 1990). In cultured keratinocytes, α_V does not form a complex with β_1 but forms an $a_V\beta_5$ heterodimer; it mediates adhesion to vitronectin, not fibronectin (Adams and Watt 1991). The β_3 subunit is not expressed in keratinocyte cultures nor at any stage of epidermal development. I did not investigate β_5 expression and therefore cannot establish whether α_V formed a complex with β_1 or β_5. However, given the heterodimer formed *in vitro*, it seems likely that the association is between α_V and β_5 in the developing epidermis.

α_V was expressed at stratification. Staining was always very weak although, like α_2 and α_5, it appeared to be elevated in developing sweat

glands. I detected α_v less consistently than the other integrins, and this may be because it was expressed at a level that was at the limit of sensitivity for the staining protocol.

3.3.6 $\alpha_6\beta_4$

β_4 has only been reported complexed to α_6, but α_6 can form heterodimers with β_1 or β_4 (Sonnenberg *et al*, 1990a). In cultured keratinocytes, α_6 is only present as $\alpha_6\beta_4$ (Carter *et al*, 1990; Klein *et al*, 1990; Adams and Watt 1991) and since the staining patterns of α_6 and β_4 were identical in the epidermis throughout development, it is likely that α_6 is associated with β_4 in the tissue. Additionally, the α_6 subunit has a higher affinity for β_4 than β_1 when both βs are present (Giancotti *et al*, 1992). The relatively weaker, more restricted staining of β_4 may may be a result of lower antibody affinity or partial masking of the epitope.

A ligand for $\alpha_6\beta_4$ has still not been conclusively identified. A polyclonal antibody to β_4 blocks adhesion of keratinocytes to laminin (De Luca *et al*, 1990), but Hall *et al* (Hall *et al*, 1990) and Sonnenberg *et al* (Sonnenberg *et al*, 1990b) report that $\alpha_6\beta_4$ is not a laminin receptor while Lee *et al* report that $\alpha_6\beta_4$ can bind laminin (Lee *et al*, 1992). $\alpha_6\beta_4$ has been reported to be a component of hemidesmosomes (Kurpakus *et al*, 1990; Stepp *et al*, 1990; Sonnenberg *et al*, 1991) and to co-localize with BPA in cultured keratinocytes (Carter *et al*, 1990); our results, as outlined below, are consistent with this conclusion. An intriguing mechanism of rapid β_4 down-regulation has been reported by Giancotti *et al*: although the extracellular domain of β_4 can only be detected in contact with the BMZ, the intracellular domain can be cleaved off and distributes throughout the basal cell cytoplasm (Giancotti *et al*, 1992). Proteolytic processing therefore allows $\alpha_6\beta_4$ functional downregulation as soon as the basal cells leave the basement membrane to differentiate.

Like $\alpha_3\beta_1$, $\alpha_6\beta_4$ was expressed prior to stratification and throughout development. Before stratification, it showed a uniform pericellular distribution but, with the onset of stratification, it became concentrated at the basal surface of the basal cells. Hemidesmosomes are reported to appear at stratification and, consistent with earlier reports (Fine *et al*, 1984;

Lane *et al*, 1985), I found bullous pemphigoid antigen (BPA) to be expressed at stratification. Double labelling of newly stratified epidermis in organ culture showed co-localization of BPA with the α_6 subunit. The sequence of expression is consistent with the idea that $\alpha_6\beta_4$ acts as a nucleating center for hemidesmosomes (Kurpakus *et al*, 1991; Quaranta and Jones 1991). However, $\alpha_6\beta_4$ cannot be limited to hemidesmosomes, because it was also expressed on the apical and lateral surfaces of basal cells and is abundant in cultured keratinocytes that form few hemidesmosomes (Bohnert *et al*, 1986).

3.3.7 Other integrins and integrin modifications

At the time this study was performed, I looked for most of the known integrins likely to be present in the epidermis. Since then, two further alpha subunits (α_7 & α_8; (Bossy *et al*, 1991; Kramer *et al*, 1991)) and two further beta subunits (β_6 (Busk *et al*, 1992) and β_7 (Sikorski *et al*, 1992; Yuan *et al*, 1992)) have been described. α_8 forms a heterodimer with β_1 and is expressed in the basal layer of chick epidermis (Bossy *et al*, 1991). β_7 is found primarily in lymphocytes, but mRNA with sequence homology to β_7 in cultured mouse keratinocytes has been reported (Yuan *et al*, 1992).

Several alternatively spliced cytoplasmic tails have been described, particularly for β_1 (Altruda *et al*, 1990; Languino and Ruoslahti 1992), but also for α_6 (Cooper *et al*, 1991; Hogervorst *et al*, 1991) and β_3 (van Kuppevelt *et al*, 1989). The variant tails are generally similarly-sized, although the consensus tyrosine phosphorylation site can be lost in the variant. No alternative functions have been ascribed to the splice variants, but this nonetheless raises the possibility that alternatively spliced forms could be expressed at different times in development. There is as yet no evidence that tail-switching occurs; the cells which express the variant tails express them as a minor proportion of total integrin.

3.3.8 Extracellular matrix proteins

Alterations in the ligands for the integrins could be expected to influence the receptor distribution. As has been reported previously,

collagen type IV and laminin were present in the BMZ throughout development (Fine *et al*, 1984); just after stratification they were also found in a pericellular distribution in the basal layer. Fibronectin was concentrated at the BMZ until about 13 weeks EGA (in agreement with observations by (Fine *et al*, 1984)). There are other ligands to which the integrins can bind, e.g. epiligrin (Carter *et al*, 1991; Rousselle *et al*, 1991), an $\alpha_3\beta_1$ and potential $\alpha_6\beta_4$ ligand, and tenascin (Bourdon and Ruoslahti 1989). It is possible that changes in the abundance of these could help account for the patterns seen.

3.3.9 Perturbation of stratification

Since the organ culture model faithfully duplicated the events of stratification, I explored the role of integrins in stratification by attempting to block stratification by specific inhibition. Since the onset of $\alpha_2\beta_1$ expression coincides with the onset of stratification, I initially cultured pre-stratification skin in the presence of monoclonal function-blocking anti-α_2. However, after overnight culture, the epidermis still stratified. One potential explanation, inability of the antibody to penetrate into the tissue, was ruled out, as shown in Fig. 13. Since the antibody has been shown to be only partially function-inhibiting (Tenchini *et al*, 1993), this has to be repeated with another antibody.

It is possible that a single integrin is not involved in stratification; it is more likely that several integrins, and non-integrins are involved. The next step would be to incubate pre-stratification skin in a cocktail of anti-integrins, or to thwart the function of several integrins using an RGD peptide, as described earlier.

3.4 Conclusions and Prospects

I have obtained indirect evidence that integrins may play an important role in establishing the spatial organization of keratinocytes during epidermal development. Several integrin subunits are expressed prior to stratification, before the earliest markers of terminal differentiation appear (Watt 1989) and the profile and distribution of

integrins changes during subsequent development, most notably at the onset of stratification.

The fact that those changes take place in organ culture offers great potential for further analysis of the role of integrins in tissue assembly. It will now be possible to use function-blocking antibodies to individual subunits in order to look at the effect on stratification, and to study whether there are environmental factors that can regulate integrin expression and the onset of stratification.

3.5 Appendix 1

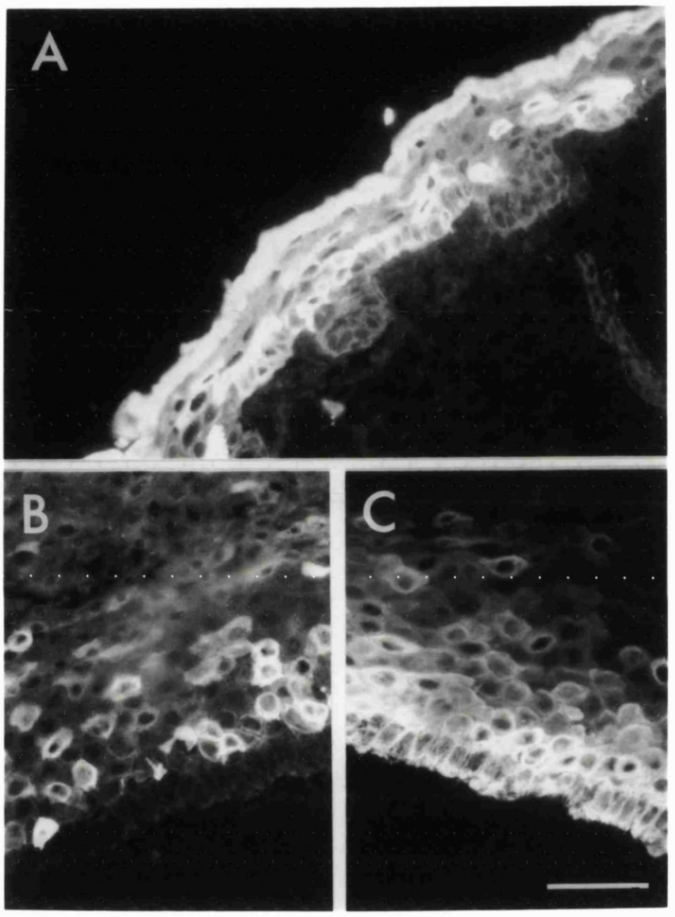
Further use of the fetal skin organ culture model — feasibility of identifying environmental and tissue inductive factors in palm and sole-specific keratin 9 expression.

3.5.1 Introduction

The epidermis of the palm (palmar epidermis) and sole (plantar) is unique in expressing keratin 9, in addition to the usual interfollicular keratins. Keratin 9 (K9), partnered primarily with K1, was first identified by Fuchs and Green (Fuchs and Green 1980), and further characterized by (Knapp *et al*, 1986; Moll *et al*, 1987; Schweizer *et al*, 1989). Rare K9-positive cells can also be detected in some other stratified squamous epithelia, usually associated with eccrine sweat gland ducts (Knapp *et al*, 1986; Moll *et al*, 1987). In other respects, palmar/plantar keratinocytes differ only in the lack of hair follicles and a thickened cornified layer. Although no definite function has been assigned to K9, it has been suggested that the increased strength of palmar and plantar epidermis, or its increased thickness, could in part be due to K9 expression (Schweizer *et al*, 1989). In the mouse, K9 expression is initiated at birth, concomitant with consistent pressure on the feet due to body weight (Schweizer *et al*, 1989). In the human, however, K9 expression is first detectable during the second trimester, well before the palms and soles are subjected to any mechanical stress (Moll *et al*, 1987). Also, cultured keratinoctes derived from the palm or sole maintain the ability to express K9 (Moll *et al*, 1987). A thicker stratum corneum, a feature of palmar/plantar epidermis, is in itself insufficient to induce K9 expression, as the thickened stratum corneum characteristic of recessive X-linked ichthyosis does not result in K9 expression (Moll *et al*, 1987). K9 is larger than most keratins, at 64-73kD — depending on species — resulting in a tail piece protruding from the tonofilament core when partnered with K1, a shorter keratin. This tail may allow binding of cytoskeletal accessory proteins such as filaggrin, further cross-linking and strengthening the epidermis (Moll *et al*, 1987).

Figure 15. Keratin 9 localization (A) *in vivo*, at 15.3 weeks EGA; and (B) keratin 9 and (C) keratin 14 after 6-week culture of embryonic skin.

Scale bar = 50 μm.


A
B
C

Keratin 9 expression might be an intrinsic property of the palmar/plantar keratinocytes (e.g. (Boukamp *et al*, 1990)), or it could be induced by the underlying dermis (Schweizer *et al*, 1984; Sharpe and Ferguson 1988; Boukamp *et al*, 1990), or basement membrane (Kurpakus *et al*, 1992). Recombination of dermal and epidermal layers is a well-established technique for the investigation of induction (Rawles 1963; Gipson *et al*, 1983; Schweizer *et al*, 1984; Bohnert *et al*, 1986). Since recombination and organ culture of fetal epidermis allows development of the epidermis to progress (Fisher and Holbrook 1987), I explored the use of human fetal skin organ culture to determine 1) if K9 expression could be induced and maintained in culture, and 2) the inductive tissue responsible for K9 expression. As well as allowing analysis of changes in integrin expression during skin development, this model allows the analysis of multiple changes associated with stratification and development.

3.5.2 Results and Conclusions

As reported previously (Moll *et al*, 1987), scattered suprabasal cells strongly expressed K9 in isolated palm epidermis by 15 weeks EGA (Fig. 15A), but K9 was not detected in tissues from other body sites. 9-week old sole did not express K9, but after culture for 6 weeks, a pattern of K9 expression similar to the 15-week epidermis could be detected (Fig. 15B), although fewer basal cells than in a 15 week fetus were positive. In this respect, the cultured epidermis more closely resembled more mature epidermis, in which no basal cells are K9 positive (Moll *et al*, 1987). Keratin 14, normally detectable in all keratinocytes, was also present (Fig. 15). These observations showed that it would be feasible to use tissue recombination to determine the tissue responsible for regulation of keratin 9 expression.

Chapter 4

Integrin expression during wound healing: co-expression of integrins and terminal differentiation markers.

4.1 Introduction

During epidermal development there are major changes in the abundance and distribution of individual integrin subunits, providing indirect evidence for a role in establishing the spatial organization of keratinocytes in the epidermis (Chapter 2 and (Hertle *et al*, 1991)). Since some of the events of epidermal development are recapitulated during epidermal wound healing, and in view of the evidence that integrins can regulate keratinocyte adhesion and terminal differentiation, I was interested to discover what happens to integrin expression when the epidermis is re-formed following wounding.

4.1.1 Epidermal wound healing

The epidermis protects the underlying tissues from the environment and mechanical damage, and prevents desiccation and infection. If damaged, the epidermis is capable of rapid and complete wound healing. Besides re-establishing the protective barrier for the body, the healed epidermis must return to its normal state from a highly dynamic state (e.g. highly proliferative, migratory, expressing ECM-degrading enzymes). These needs result in a complex sequence of events (reviewed in (Clark 1989)) which allows wounds to be a factor in in problems as diverse as ulcers (Grinnell *et al*, 1992) and viral tumorigenesis (Dolberg *et al*, 1985; Sieweke *et al*, 1990).

Wound healing requires invasion by cells of the immune system and their attendant cytokines; followed by stimulation of keratinocyte migration and proliferation; degradative, contractile, and synthetic activity by the dermal fibroblasts; and angiogenesis (Odland and Ross 1968; Clark 1989; Grinnell 1990; McKay and Leigh 1991). Once the wound has closed, the keratinocytes must return to their former state. Hyperproliferation must be quickly down-regulated, through attenuation of stimulatory cytokine signals, to prevent a buildup of cells and excessive scarring. Contact inhibition and the release of cytostatic cytokines (e.g. TGF-β (Mansbridge and Hanawalt 1988)) in the correct context are likely modulators of hyperproliferation.

4.1.1.1 Coagulation and inflammation

The earliest event in wound healing is the inflammatory response — essential to combat infection — evident within minutes (reviewed in (Clark 1989)). Activated or damaged platelets release their contents into the wound area, initiating blood clotting and attracting neutrophils and monocytes (which differentiate *in situ* to macrophages), in turn releasing additional cytokines. Cytokines are essential to and closely involved in wound healing; multiple cytokines and complex interactions come into play (Lynch *et al*, 1989) and will be discussed in more detail in the next chapter.

The infiltration of immune cells into the wound involves several classes of adhesion molecules (reviewed by (Albelda and Buck 1990)) and multiple cytokines (reviewed by (Nathan and Sporn 1991)). Cytokines such as interferon-γ and interleukin-1β induce leukocytes to express LFA-1 (lymphocyte function-associated antigen; a β_2 integrin (Sanchez-Madrid *et al*, 1983)), allowing adhesion of the leukocytes to the inflamed endothelium, followed by extravasation into the wound area (Norris 1990). On cytokine induction, keratinocytes express ICAM-1 (intercellular adhesion molecule-1; an immunoglobulin superclass adhesion molecule), a ligand for LFA-1 (Caughman *et al*, 1990). ICAM-1/LFA interactions allow adhesion to the keratinocytes and targeting of the leukocytes to the wound (McKay and Leigh 1991). The importance of leukocyte adhesion in wound healing is illustrated by the wound healing impairment (slowed and/or incomplete) in patients with lymphocyte adhesion deficiency (LAD), stemming from a mutation resulting in absence of β_2 expression which prevents accumulation of granulocytes, monocytes, and lymphocytes at the wound (Anderson and Springer 1987; Hibbs *et al*, 1990). The lack of inflammatory cytokine contribution in LAD patients appears to be the key factor in impaired wound healing, preventing e.g. neovascularization.

A fibrinogenous clot, necessary to prevent blood loss and protect the exposed dermis, forms within minutes of wounding. The clot both provides a provisional matrix for migration and an obstacle to the migrating keratinocytes (Clark 1989). The keratinocytes migrate through the clot, rather than underneath it, by releasing proteases (Porras-Reyes *et*

al, 1991; Rømer *et al*, 1991), possibly triggered by integrin signal transduction (Werb *et al*, 1989).

4.1.1.2 Keratinocyte migration

Re-epithelialization — keratinocyte migration and proliferation to close the wound — proceeds both from the edges of the wound and from the associated structures of the epidermis (adnexae: sweat glands and hair follicles), and generally begins within 24-48 hours following wounding (Winter 1962; Odland and Ross 1968; Ortonne *et al*, 1981; Clark 1989). The new keratinocytes originate primarily from hair follicles, especially in superficial wounds (Donaldson and Mahan 1988). Migration may be stimulated by TGF-β, suggested by its ability to increase fibronectin synthesis and deposition *in vitro* (Nickoloff *et al*, 1988; Wikner *et al*, 1988), and is also stimulated by TGF-α and EGF *in vitro* (Barrandon and Green 1987a). Although the keratinocytes of the hair follicle are different from interfollicular keratinocytes in several respects (e.g. constitutive expression of K6/K16 (Stark *et al*, 1987)), they share many features with interfollicular keratinocytes, perhaps representing a "less differentiated" subpopulation, able to take on the interfollicular keratinocyte phenotype out of the follicle environment (Lenoir *et al*, 1988; Stark *et al*, 1990).

Several cytoskeletal proteins undergo migration and wound healing-related changes (also discussed below); for example, additional keratins are expressed. Keratin 6 and its partner K16 are frequently a marker of hyperproliferative keratinocytes — their function is unknown — expressed in cultured cells (Roop *et al*, 1987), wounds (Schermer *et al*, 1989; Lane *et al*, 1992b), and diseases characterized by hyperproliferation such as psoriasis (Weiss *et al*, 1984). A preliminary report indicates that expression may be induced by TGF-β, an inflammatory cytokine (Ladin and Morhenn 1991). Keratins 4 and 13 (characteristic of parakeratinizing and oral epithelia (van Muijen *et al*, 1984)) are also transiently expressed, just under the blister roof (Lane *et al*, 1992b).

4.1.1.2.1 Mechanisms of keratinocyte migration

In normal epidermal differentiation, keratinocyte movement is limited to that required for differentiation, i.e. upward. During wound healing, rapid lateral migration — stimulated at least in part by TGF-β (Nickoloff *et al*, 1988) and TGF-α (Barrandon and Green 1987a) — is essential to healing the wound. Within hours of wounding, keratinocyte migration to close the wound is initiated. There is some controversy over the actual mechanism of migration in closing the wound, though. The prevalent hypothesis, the so-called rolling or tractor-tread mechanism, postulates that keratinocytes at the leading edge "leap-frog" each other (Krawczyk 1971; Ortonne *et al*, 1981; Hunt 1990). The keratinocyte behind the leading one migrates over the top of the leading keratinocyte, and adheres. Another trailing keratinocyte migrates over it, and the process continues until the wound is closed. This hypothesis is supported by the expression of keratin 10, a suprabasal marker, by all keratinocytes at the leading edge and the initial absence of melanocytes in the migrating epidermis (Ortonne *et al*, 1981), suggesting that the cell sheet is not simply moving towards the center of the wound. This model requires keratinocytes to co-express features of both differentiated (i.e. suprabasal) and undifferentiated (i.e. basal) keratinocytes.

The other hypothesis, the "sliding" mechanism, requires the migrating keratinocytes to move as a sheet, with leading keratinocytes migrating over the ECM and pulling the trailing keratinocytes behind. Both types of migration are possible; the mechanism used may be species-dependent (Donaldson and Mahan 1988). The sliding mechanism has been demonstrated, at least in amphibian skin; the rolling mechanism has yet to be unequivocally proven.

In both of these models, proliferation is not immediately necessary for migration to occur (Odland and Ross 1968; Sherratt and Murray 1991; Sarret *et al*, 1992). Initiation of migration precedes an induction of proliferation and can occur independently (Matoltsy 1960; Etoh *et al*, 1974; Ortonne *et al*, 1981). It has been shown that the basal cells can become flatter, allowing the available keratinocytes to cover a greater area (Folkman and Moscona 1978). A mathematical model for wound healing, based on experimental data, is consistent with an initial lag in

proliferation, followed by stimulation (Sherratt and Murray 1991). Stimulation of proliferation is essential to ultimately supply the cells to cover the wound, and is highest at the migrating edge, where — paradoxically — TGF-β expression is highest (Kane *et al*, 1991). The protected site of adnexal structures (deep within the dermis) provides a source of keratinocytes and has led some to suggest that the stem cells may reside there (Lenoir *et al*, 1988; Hall and Watt 1989; Cotsarelis *et al*, 1990). Expression of K19 in the hair follicle outer root sheath is suggestive of at least pluripotent progenitor cells, though probably not stem cells (Bartek *et al*, 1985; Stasiak *et al*, 1989).

4.1.1.2.2 Migrating keratinocyte interaction with the extracellular matrix

Wounds can be divided into two categories, based on whether the migrating keratinocytes are exposed to the dermis or not. In deep wounds, the basement membrane is destroyed and the keratinocytes are exposed to an unaccustomed environment (the dermis) and forced to migrate over a "provisional" fibronectin matrix (Grinnell *et al*, 1981; Clark 1990). During wound healing in the rat, the form of fibronectin expressed in the wound is identical to that found during embryogenesis, suggesting that this alternatively spliced, embryonic form may be more appropriate for proliferation and migration than the adult form (ffrench-Constant *et al*, 1989). Also, the dermal fibroblasts become directly involved in deep wound healing (discussed below). After the keratinocytes have closed the wound, a new basement membrane is synthesized by the keratinocytes and fibroblasts, starting at the wound margin and moving towards the center of the wound (Stanley *et al*, 1981a; Clark *et al*, 1982; Donaldson and Mahan 1988).

In the other category of wound, superficial or partial thickness, the basement membrane is not damaged sufficiently to expose the keratinocytes to the dermis — the major components of the lamina densa remain on the dermis (Hunter *et al*, 1974; Lane *et al*, 1992b). This type of wound can be induced experimentally by suction or naturally by e.g. burns to create a blister. The keratinocytes migrate over an ECM to which they are accustomed (the basement membrane) and are not exposed to the dermis, although they may be exposed to serum fibronectin in the blister

fluid (Wysocki and Grinnell 1990). These wounds heal rapidly, but probably not because migration is more rapid than in deep wounds: laminin, a basement membrane component, inhibits keratinocyte migration (Donaldson and Mahan 1988; Nickoloff *et al*, 1988; Kim *et al*, 1992b). Inflammation and tissue damage are minimized, which may increase the speed of healing.

There has been some work on keratinocyte cytoskeletal changes during wound healing, e.g. no change in actin or vinculin (Mansbridge and Knapp 1987); an increase in cytoplasmic myosin and decrease in desmosomes (Gabbiani *et al*, 1978). Recent work in our laboratory on the same stages as presented below indicated that talin and vinculin were absent at the leading edge of migration, but still faintly present at the outer margins of the wound (Kubler & Watt, submitted). Localization of gelsolin, filamin, and α-actinin was normal at the outer margins of the wound, but dereased in intensity at the migrating edge. Both filamin and α-actinin, normally at the cell margins, were more diffuse at the migrating edge. Actin staining was essentially normal at all stages, as was staining for talin, vinculin, gelsolin, filamin, and α-actinin after wound closure.

Changes in the interactions of integrins with the substrates (e.g. loss of focal contact adhesion) on migrating cells have been extensively studied in culture (Mansbridge and Knapp 1987; Akiyama *et al*, 1989; Grinnell 1990; Yamada *et al*, 1992). Keratinocytes can migrate on fibronectin, collagen, and thrombospondin (O'Keefe *et al*, 1985; Nickoloff *et al*, 1988; Woodley *et al*, 1988; Guo *et al*, 1990), although laminin (Nickoloff *et al*, 1988; Woodley *et al*, 1988) and vitronectin (Brown *et al*, 1991) inhibit migration. Specific integrins can mediate migration on specific substrates. $\alpha_5\beta_1$, weakly expressed on freshly isolated keratinocytes, is upregulated in culture, concomitant with increased migration on fibronectin (Guo *et al*, 1990). RGD peptides, which represent the cell-binding site of fibronectin and can inhibit $\alpha_5\beta_1$ function, inhibit migration on fibronectin (Kim *et al*, 1992a; Kim *et al*, 1992b). An antibody to α_2, but not α_5, inhibits migration on collagen (Kim *et al*, 1992b). Inhibition of $\alpha_3\beta_1$, a laminin (Ignatius and Reichardt 1988; Hall *et al*, 1990) and epiligrin (Carter *et al*, 1991; Rousselle *et al*, 1991) receptor, promotes migration on other substrates. Adhesion to laminin thus appears to inhibit migration (Donaldson and Mahan 1988; Nickoloff *et al*, 1988; Kim *et al*, 1992b). Given the altered cell-cell and cell-

ECM interactions, and a cytokine-rich environment, modulation of integrins — the main class of adhesion molecules — would be anticipated during wound healing.

One of the adhesive structures through which keratinocytes interact with the basement membrane is hemidesmosomes. These structures, whose components extend from the cytoskeletal intermediate filaments into the dermis and include $\alpha_6\beta_4$ (Carter *et al*, 1990; Kurpakus *et al*, 1990; Stepp *et al*, 1990), are disrupted during migration (Krawczyk 1971; Gabbiani *et al*, 1978; Ortonne *et al*, 1981; Donaldson and Mahan 1988). The re-assembly of hemidesmosomes during migration has been studied both *in vivo* and *in vitro*; but whether the leading cell can assemble hemidesmosomes is still controversial (see Discussion). However, the sequence of assembly has been reported in an *in vitro* explant model. $\alpha_6\beta_4$ is suggested to have a central role in both hemidesmosome assembly — ventral localization is required for hemidesmosome formation — and maintenance, suggested by hemidesmosome dissasembly by an α_6 antibody (Kurpakus *et al*, 1991).

4.1.1.3. Fibroplasia and angiogenesis

Several days after creation of a deep wound, fibroblasts migrate into the wound area and their proliferation is stimulated. Fibroblasts are probably attracted to wounds by chemotactic factors, possibly those released by platelets and macrophages (Madtes *et al*, 1988). Fibroblasts form the granulation tissue, composed primarily of fibronectin, but also collagen (Grinnell *et al*, 1981). Wound contraction — possibly stimulated by PDGF from platelets and monocytes (Clark *et al*, 1989) — which accelerates wound healing and re-epithelialization by bringing together the edges of the wound, has been postulated to be mediated by "myofibroblasts", actin-rich fibroblasts found along wound contraction lines (Clark 1989). Contraction is probably mediated by $\alpha_2\beta_1$ integrin (Klein *et al*, 1991; Schiro *et al*, 1991). Remodeling of the dermal matrix continues well after the wound is closed (Clark 1989).

Capillary sprouts, led by endothelial cells probably stimulated by fibroblast growth factor (Folkman and Klagsbrun 1987) invade the dermal

wound area several days after wounding (reviewed in (Clark 1989)) and ultimately join to vascularize the former wound area. TGF-β-stimulated fibronectin synthesis by endothelial cells encourages migration of capillary buds (Madri *et al*, 1988).

4.1.2 Approach

Although several types of wound models could have been used (reviewed in (Cohen and Mast 1990)), I used that of re-epithelialization following suction blister formation (Kiistala and Mustakallio 1967; Lowe and van der Leun 1968; Hunter *et al*, 1974; Lane *et al*, 1992b) since it involves damage to the epidermis only. Application of vacuum to a small area of skin causes a blister to form, in which the epidermis detaches from the dermis, but the basement membrane remains essentially intact (Hunter *et al*, 1974). I examined the patterns of expression of a range of integrin subunits at different times after blistering and attempted to correlate the results with expression of three integrin ligands and three markers of keratinocyte terminal differentiation. I find evidence that the changes in integrin expression that are seen at the time of wound closure are also found in involved psoriatic lesions. These results raise a number of intriguing questions about integrin function and the factors that regulate integrin expression in hyperproliferative epidermis, which are explored in the next chapter.

4.2 Results

4.2.1 Normal epidermis

Sections of human foreskin were stained at the same time as the suction blister specimens, and acted as positive controls (not shown). In normal epidermis integrin expression is largely confined to the basal layer: β_1, its a partners and α_v have a uniform pericellular distribution, although staining for α_v and α_5 is weaker and more diffuse than for the other subunits. α_6 and β_4 also have a pericellular distribution in the basal layer, but show a relative concentration at the basement membrane zone (Chapter 2; (Hertle *et al*, 1991)).

4.2.2 Healing suction blisters

4.2.2.1 1 day after blister formation

A schematic model of the healing blister is shown in Figure 1. The staining pattern 1 day after blistering is shown in Figure 2. The epidermis that remained attached to the dermis at the blister edges had the same distribution of integrins as in normal, undamaged epidermis (not shown). Integrin-positive keratinocytes within the blister roof were not organized as a single basal layer, but instead suprabasal clusters of cells expressing integrins were observed (Fig. 2 B-G). This distribution was observed for all integrin subunits tested (α_2, α_3, α_5, β_1, α_v, α_6, β_4). Most of the cells within the blister roof that were expressing α_6 and β_4 showed punctate intracellular staining in addition to cell surface staining (arrows in Fig. 2 F, G). The blister roof persisted for 3-4 days and during this time the number of positive clusters decreased, and more individual positive cells were observed.

4.2.2.2 2 days

By 2 days lateral migration of keratinocytes from the edges of the wound and from hair follicles within the wound was underway

(Text continues on p. 181)

Figure 1 Schematic illustration of the suction blister model. Arrows indicate migrating edges of the epidermis; a central hair follicle is shown.

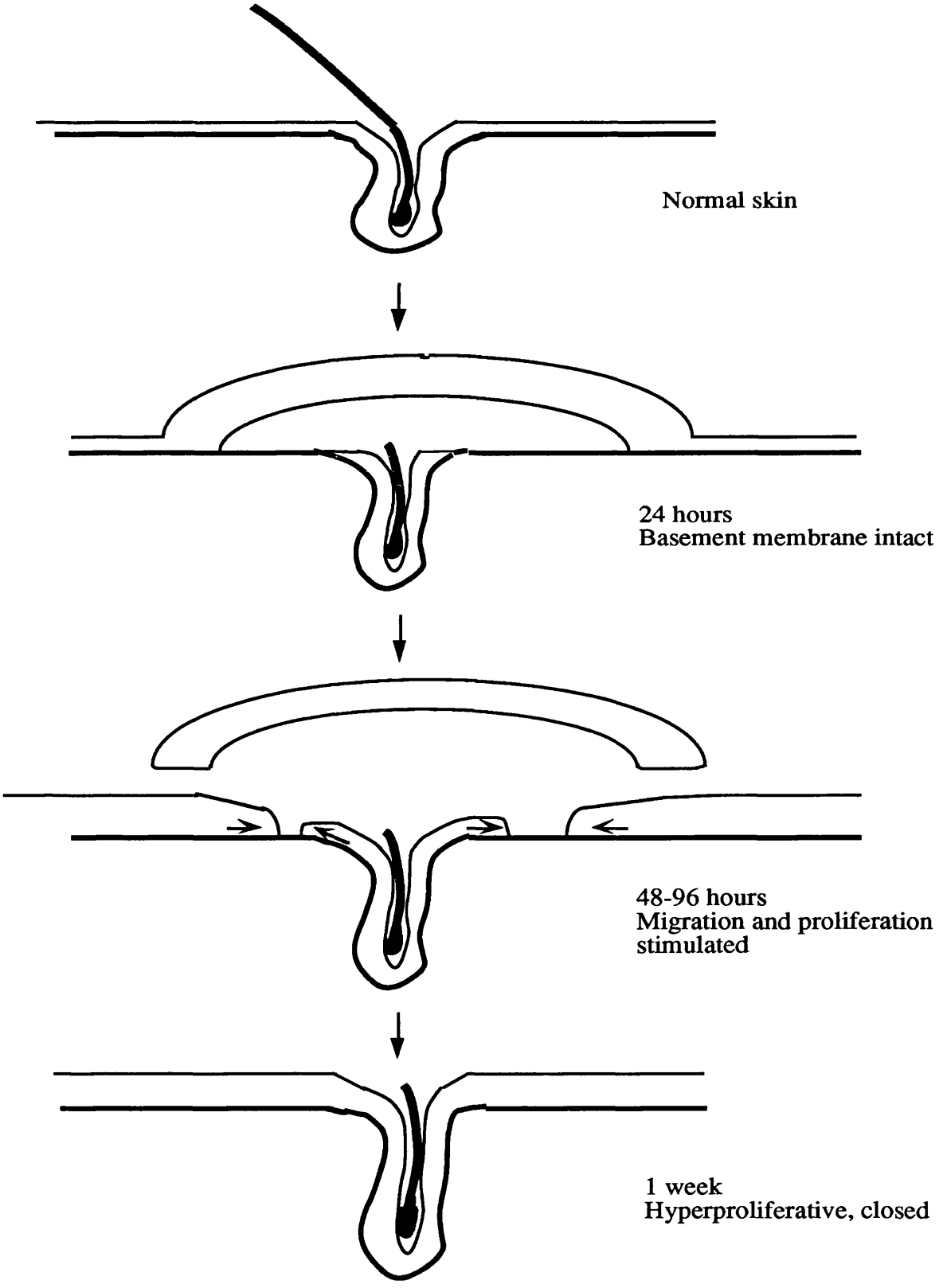

Normal skin
24 hours
Basement membrane intact
48-96 hours
Migration and proliferation
stimulated
1 week
Hyperproliferative, closed

Figure 2 Blister roof, 1 day after wounding. A: phase contrast micrograph of blister roof and attached, unwounded epidermis at right. B-G: stained with antibodies to the integrin subunits indicated. Note suprabasal clusters of positively stained cells. Arrows in F and G indicate punctate intracellular staining. H: phase contrast micrograph of F. Note: B and C are unintentionally the same print, but the result is valid — staining was identical for both subunits.

Scale bars = 50 μm.

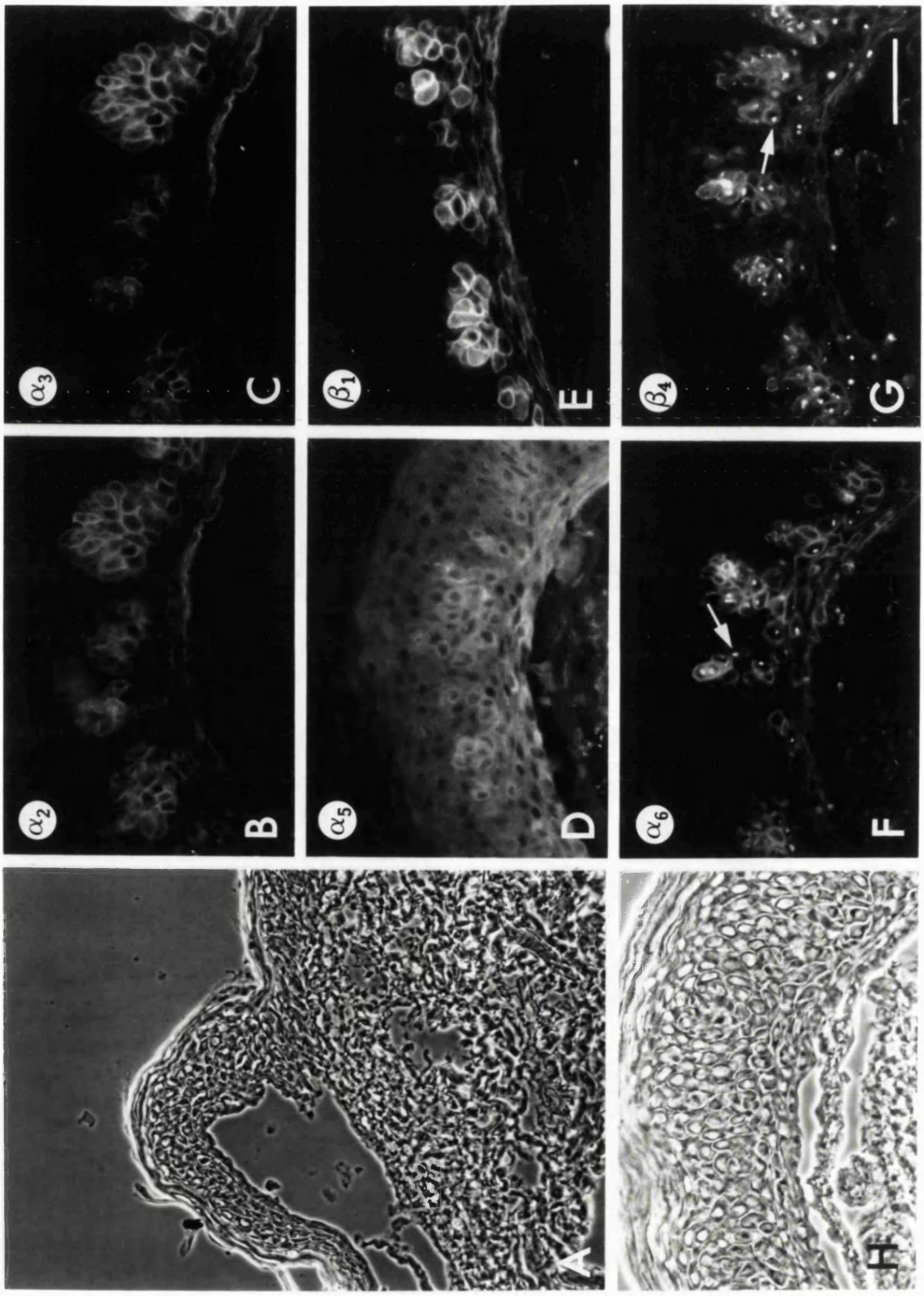

A
B
C
D
E
F
G
H
α2
α3
α5
α6
β1
β4

Figure 3 2 days after wounding. A, C, D: stained with antibodies to the integrin subunits indicated. B: phase contrast micrograph of A. Keratinocytes are migrating towards the right in A and B. In D keratinocytes are migrating towards the center from both sides of the field. Arrows indicate the leading edges. There is some non-specific staining or staining of membrane fragments on the exposed basement membrane.

Scale bar = 50 μm.

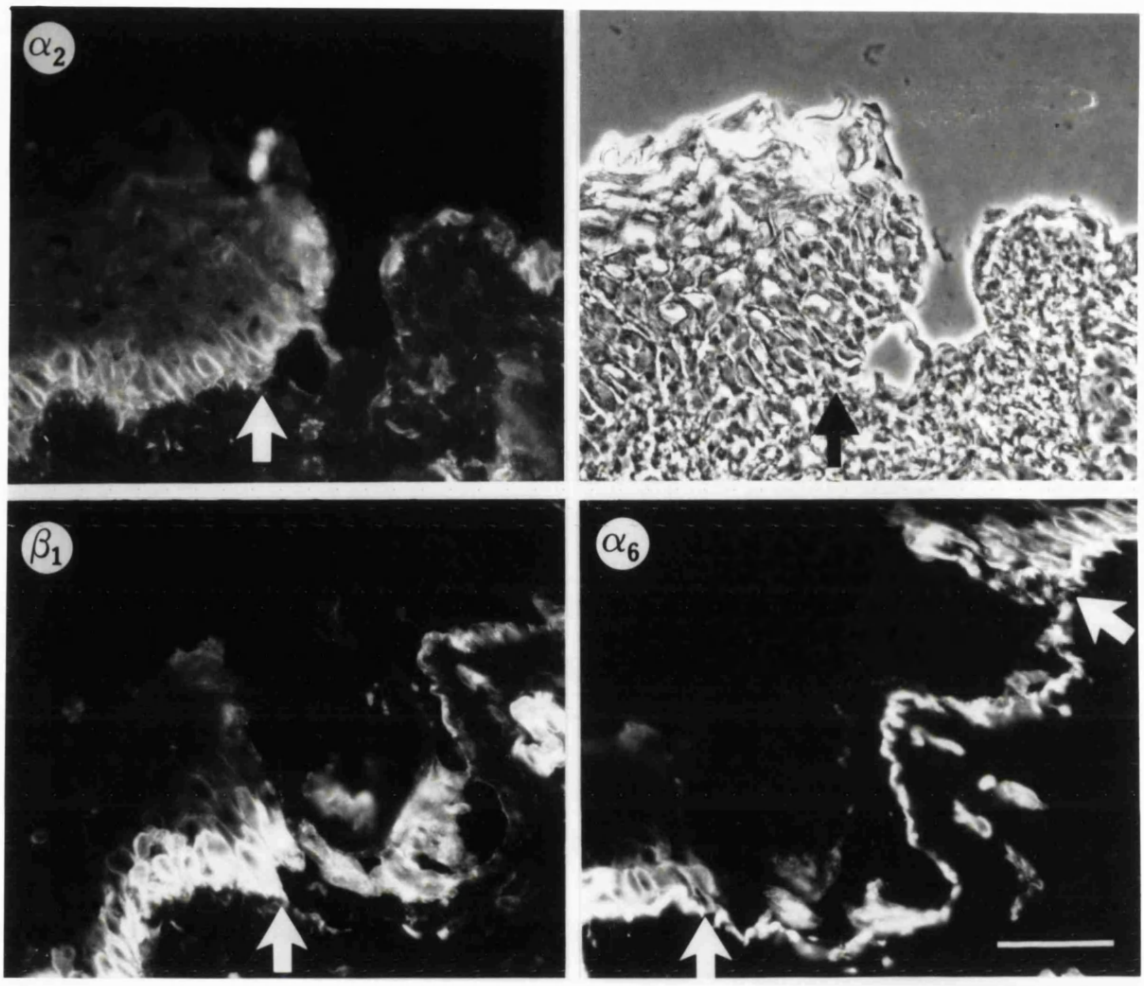

α_2
β_1
α_6

Figure 4 3 days after wounding. A, C: stained with antibodies to the integrin subunits indicated. B, D: phase contrast micrographs of A, C respectively. Keratinocytes are migrating towards the right. Note that there is suprabasal staining in C but not in A.

Scale bar = 50 μm.

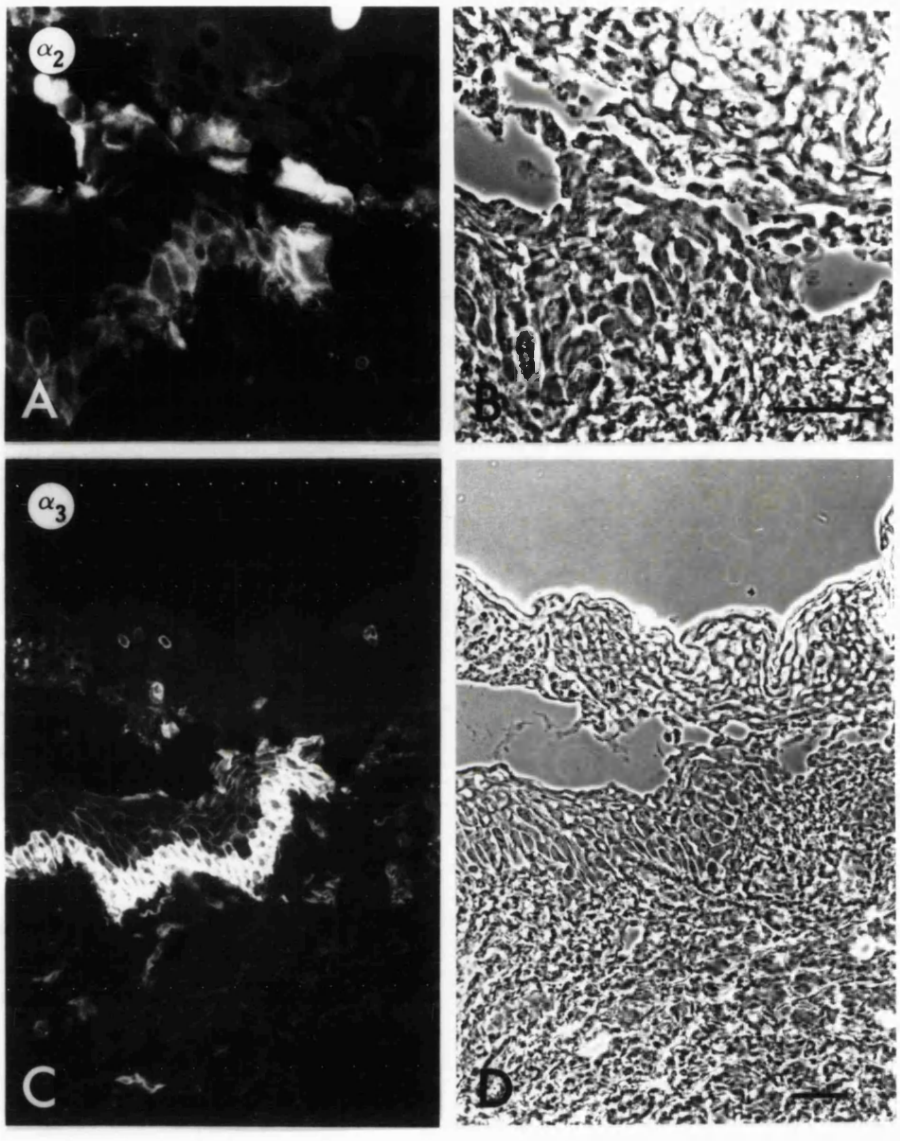

α_2
A
B
α_3
C
D

Figure 5 4 days after wounding; the wound is fully closed in most cases. A, B: stained with antibodies to the integrin subunits indicated; note appearance of suprabasal staining in B. C: phase contrast micrograph of B; the blister roof can still be seen at the top.

Scale bar = 50 μm.

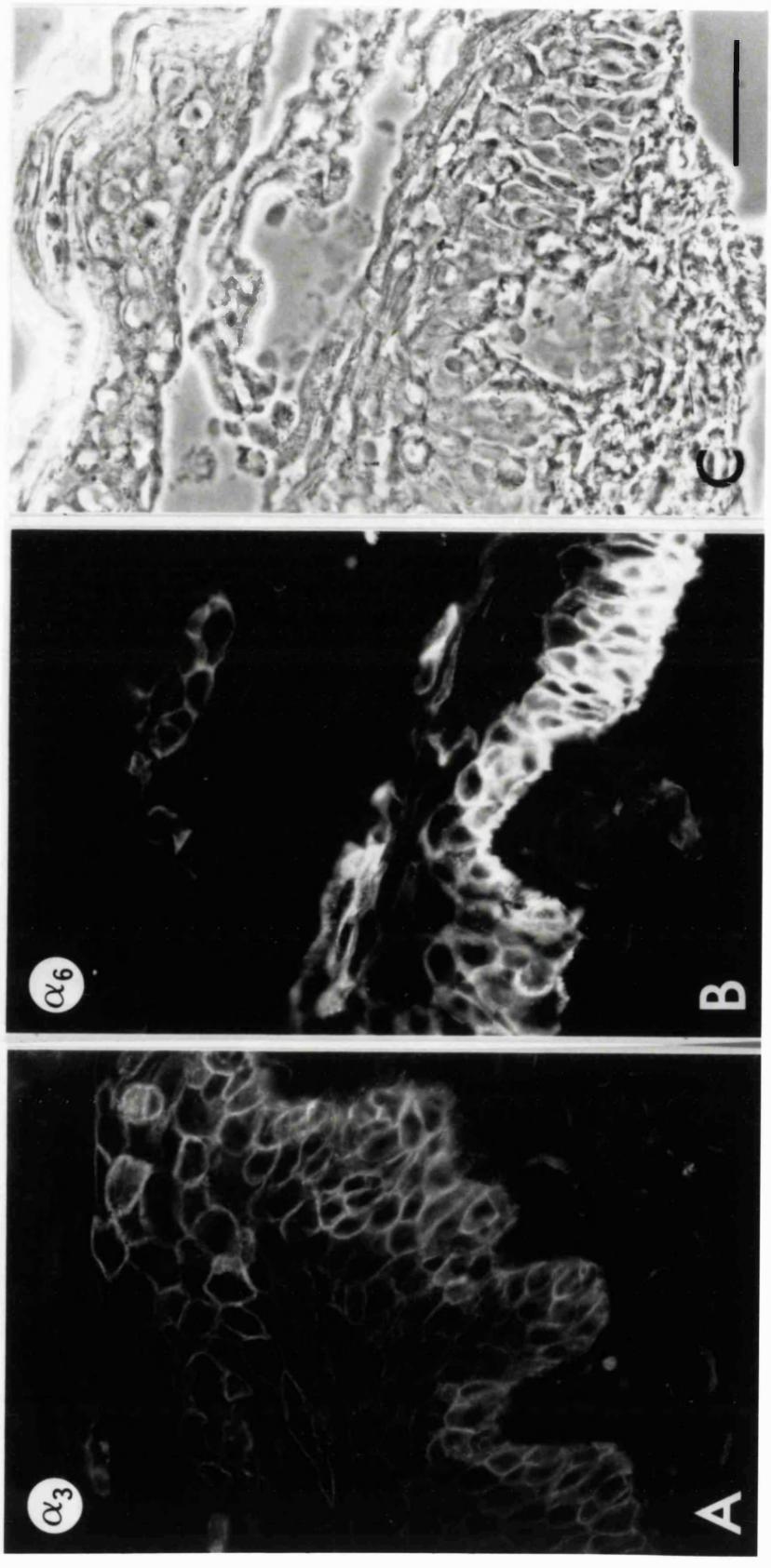

α3
A
α6
B
C

Figure 6 7 days after wounding. A, B, D-I: stained with antibodies to the integrin subunits indicated. C: phase contrast micrograph of B. H: edge of wound. I: note disorganized cell layers.

Scale bars in H and I = 50 μm; A-H are at the same magnification.

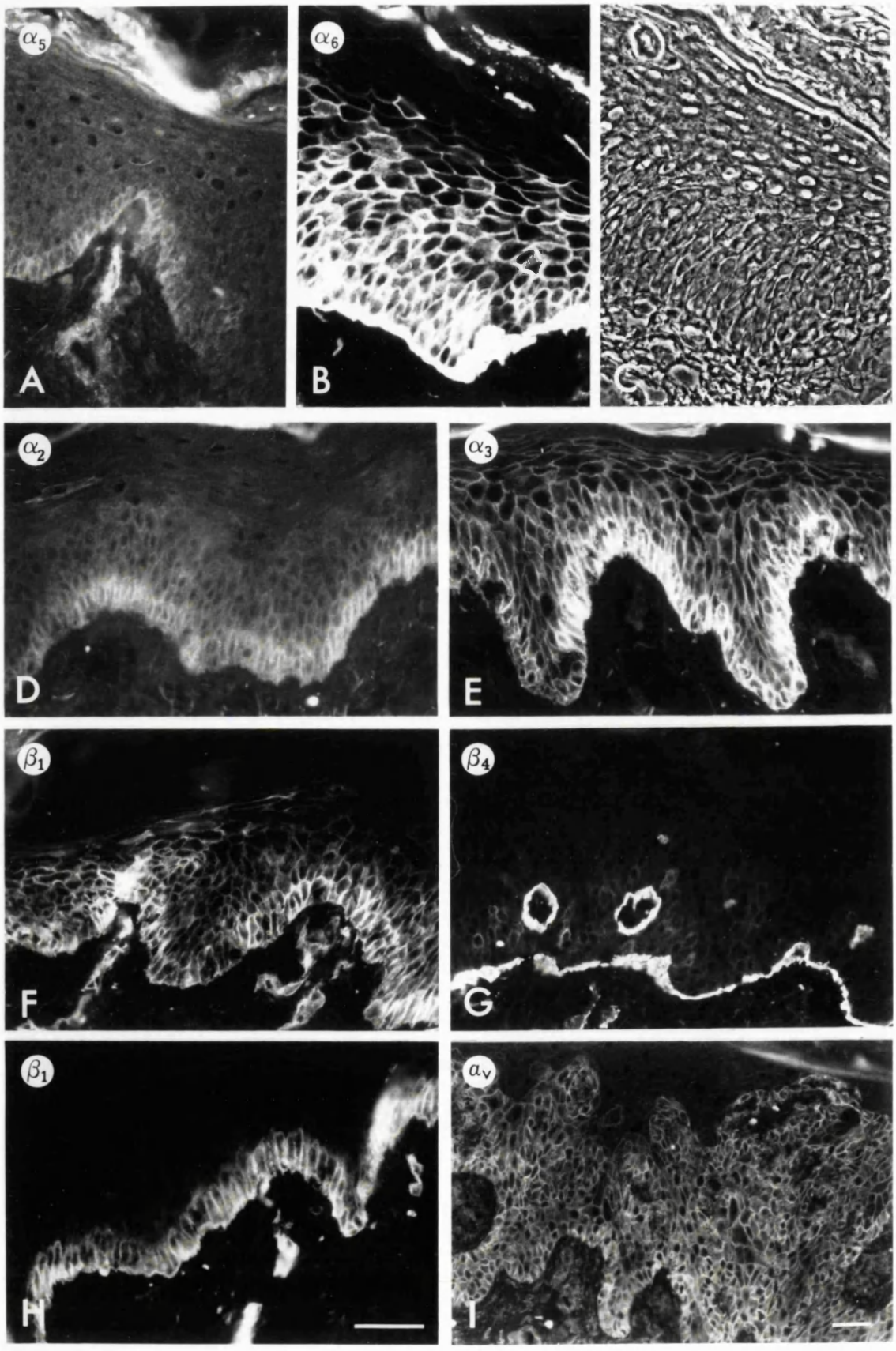
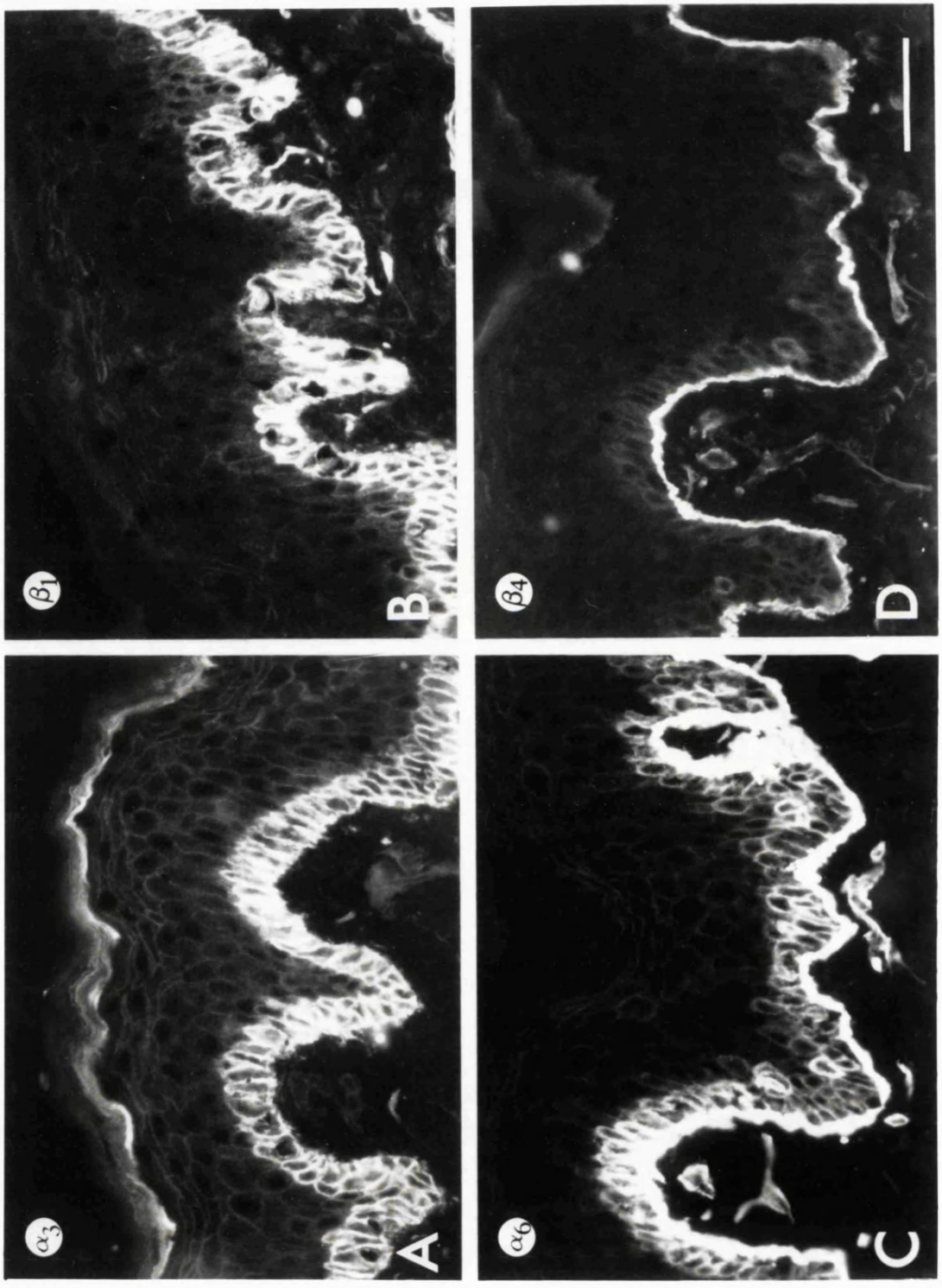

α_5
A
α_6
B
C
α_2
D
α_3
E
β_1
F
β_4
G
β_1
H
α_v
I

Figure 7 2 weeks after wounding. A-D: stained with antibodies to the integrin subunits indicated.

Scale bar = 50 μm.


α3
A
β1
B
α6
C
β4
D

(Kiistala and Mustakallio 1967; Ortonne *et al*, 1981; Lane *et al*, 1992b), although a tongue of migrating keratinocytes was not always clearly visible (e.g. Fig. 3A). The staining pattern of the migrating keratinocytes was the same as in normal, undamaged epidermis: all subunits were confined to the basal layer and the cellular distribution of each integrin was normal (Fig. 3). Some staining of the denuded basement membrane was also detected (for example, Fig. 3 C, D), possibly reflecting staining of basal cell membrane fragments that had been left behind (Hunter *et al*, 1974). No changes in integrin expression were observed distal to the migrating wound edge (not shown).

4.2.2.3 3 days

By 3 days faint suprabasal staining for α_3 (Fig. 4 C) and β_1 (not shown) was seen at the leading edge of the migrating epidermis. Within 1mm from the leading edge α_3 and β_1 were confined to the basal layer. The staining patterns of all the other integrin subunits were unchanged at 3 days (see, for example, Fig. 4 A).

4.2.2.4 4 days

By 4 days, wound closure had occurred in most specimens examined. α_6, in addition to α_3 and β_1, was expressed in suprabasal cells at the center of the wound (Figure 5). As before, the staining pattern distal to the wounds was normal and all the other integrin subunits, including β_4, were confined to the basal layer at the site of the wound.

4.2.2.5 6-7 days

6-7 days after blistering, all of the wounds had re-epithelialized, but the epidermis was hyperproliferative: it was thicker than normal epidermis and the basal cells were vertically elongated (Fig. 6C and (Lane *et al*, 1992b)). α_2, α_3, α_6 and β_1 were found in all the living cell layers at the center of the wound (Fig. 6). The strong pericellular staining suggested

that the integrins were present at the cell surface and not accumulated intracellularly. Suprabasal expression extended almost to the edge of the biopsies, declining gradually to basally restricted staining (Fig. 6 H). Suprabasal α_v was weak or absent on all but one specimen, in which the suprabasal cell layers were highly disorganized and staining was strong compared with normal epidermis (Fig. 6 I). α_5 remained confined to the basal layer (Fig. 6 A), and β_4 stained the basal and first suprabasal layers (Fig. 6 G).

4.2.2.6 2 weeks

2 weeks after wounding the epidermis was morphologically normal (Fig. 7 and (Lane *et al*, 1992b)) and expression of all integrin subunits was once more largely confined to the basal layer (Fig. 7 and results not shown). The intensity of staining appeared somewhat greater than in normal skin.

4.2.2.7 Extracellular matrix proteins

Biopsies taken 1 week after wounding were stained with antibodies to fibronectin, type IV collagen and laminin, to see whether these integrin ligands were, like their receptors, present in the suprabasal layers. All three proteins were present in the dermis and basement membrane zone (Fig. 8), in the same distribution as in unwounded epidermis (Chapter 2; (Hertle *et al*, 1991)). They were absent from the suprabasal layers, apart from some areas where the keratinocyte layers were disorganized and faint, diffuse staining was observed (Fig. 8 B, C; compare to integrin staining in Fig. 6 I).

4.2.2.8 Terminal differentiation markers

The suprabasal integrin staining observed 1 week after blistering suggested that keratinocytes were expressing integrins while undergoing terminal differentiation. Three markers of terminal differentiation were therefore examined: keratin 10, which is expressed by all suprabasal cells in

Figure 8 Extracellular matrix proteins, one week after wounding. A: fibronectin; B: collagen type IV; C: laminin. Staining in B and C is not extracellular.

Scale bar = 50 μm.

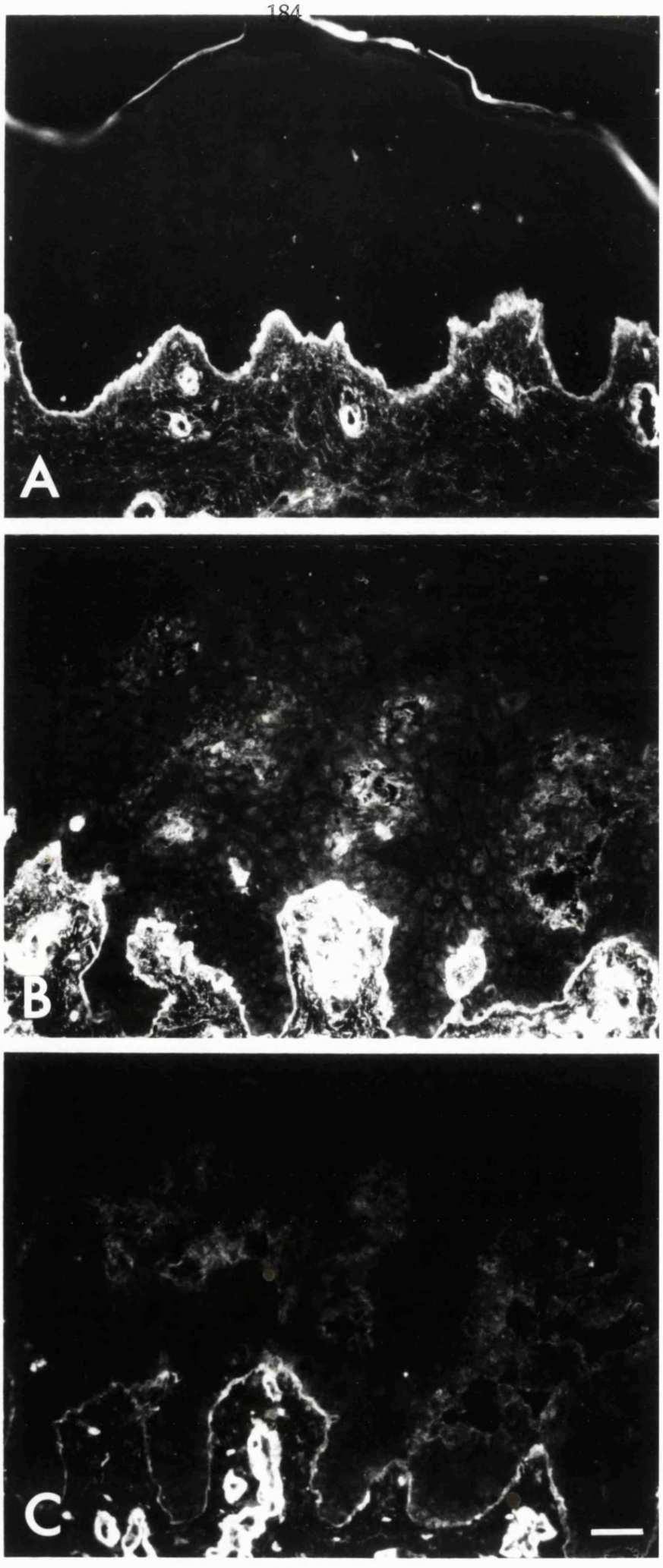
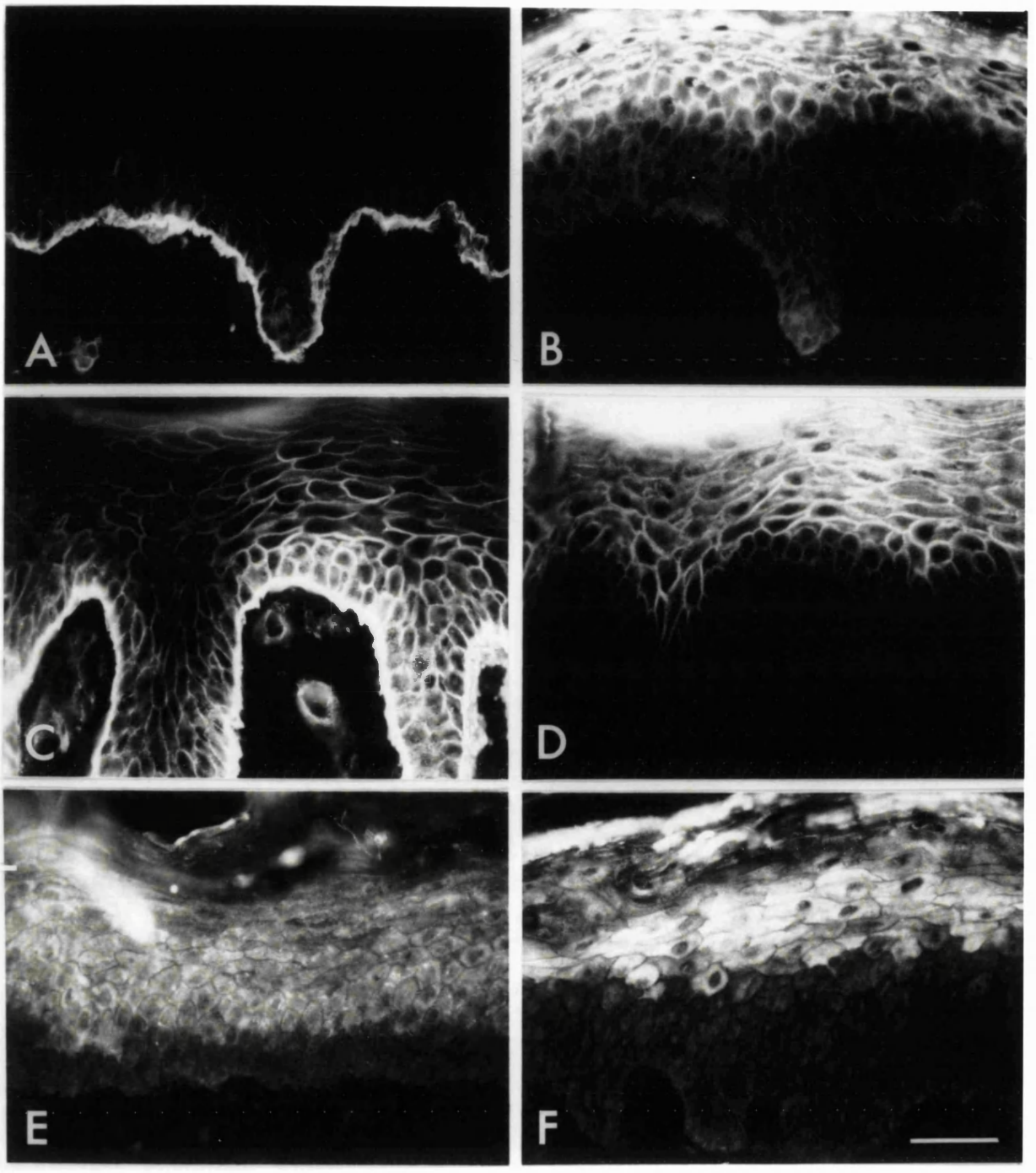

A
B
C

Figure 9 Terminal differentiation markers, one week after wounding. A-D: Dual labelling for α_6 (A, C) and involucrin (B, D). A, B: distal to wound center; C-H: center of wound. E: keratin 10; F: keratin 16.

Scale bar = 50 μm.


A
B
C
D
E
F

normal epidermis (Fuchs and Green 1980; Galvin *et al*, 1989); keratin 16, which is expressed suprabasally in hyperproliferative epidermis (Weiss *et al*, 1984; Stoler *et al*, 1988; Lane *et al*, 1992b); and involucrin, a precursor of the cornified envelope. Involucrin is normally first expressed in the upper spinous layers of the epidermis, but expression begins in the lower spinous layers under hyperproliferative conditions (Fig. 9 B, D; (Bernard *et al*, 1986; Watt *et al*, 1987; Lane *et al*, 1992b). Keratins 10 and 16 were expressed in the suprabasal cell layers 1 week after wounding (Fig. 9 E, F; see also (Lane *et al*, 1992b)). Involucrin expression began in the lower spinous layers and double label immunofluorescence showed co-expression of involucrin with α_6 (Fig. 9 C, D) and β_1 integrin subunits (not shown) suprabasally. Distal to the center of the wound, involucrin expression began higher in the spinous layers, and the integrins were confined to the basal layer: the involucrin-positive cells were integrin-negative (Fig. 9 A, B).

4.2.3 Psoriasis

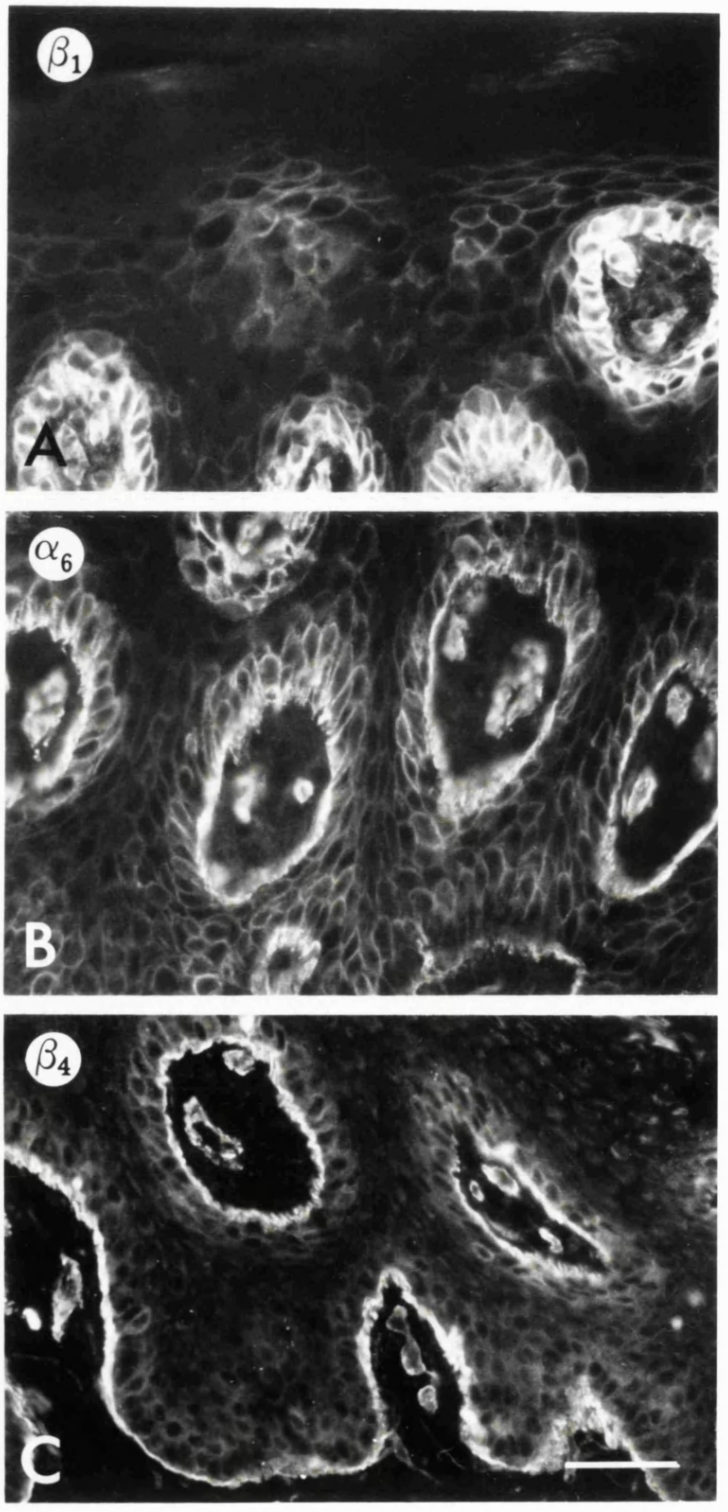
The morphological appearance of the epidermis 1 week after wounding, together with K16 expression and premature involucrin expression, indicated that the tissue was hyperproliferative. In order to discover whether suprabasal integrin expression is peculiar to wound healing or may be a more general feature of hyperproliferative epidermis, I stained sections of involved psoriatic lesions. There was suprabasal staining for two of the three subunits examined, α_6 and β_1, while β_4 remained basally restricted (Fig. 10). In contrast to wounded epidermis, however, the suprabasal staining in psoriatic epidermis was confined to discrete patches of cells (Fig. 10). Expression of involucrin was found in the lower spinous layers (not shown), co-expressed with α_6 and β_1, as found at 6-7 days in the wound.

4.2.4 Is rapid stratification capable of inducing suprabasal integrin expression?

It is possible that the suprabasal staining — at least in the immediate suprabasal cells — I observed was due to the rapid stratification

Figure 10 Involved psoriatic lesion stained with antibodies to the integrin subunits indicated. Note patches of suprabasal staining in A and B.

Scale bar = 50 μm.


β_1
A
α_6
B
β_4
C

occurring in the wound. Keratinocytes grown *in vitro* in very low levels of calcium do not stratify, due to their inability to form calcium-dependent desmosomes (Yuspa *et al*, 1989). However, on addition of calcium, stratification is rapid, occurring within 24 hours (Magee *et al*, 1987). Since keratinocytes *in vitro* do not express integrins suprabasally, I induced rapid stratification by switching keratinocytes grown in low calcium medium (0.15mM) into normal medium (containing calcium at 1.2mM) and screened for suprabasal integrin expression. Integrin localization was examined on cell sheets detached using dispase at 1-4 days post-calcium addition (Fig. 11). At all timepoints (not shown) integrins were not expressed suprabasally. Therefore, the integrin expression pattern was identical to that observed in keratinocytes stratified at the normal level of calcium, i.e. integrin expression was confined to the basal layers.

4.2.5 Is retinoic acid capable of inducing suprabasal integrin expression?

It has been previously reported that treatment of cultured keratinocyte raft cultures with retinoic acid — at greater than physiological levels — could induce $\alpha_5\beta_1$ expression suprabasally (Asselineau et al, 1989). I treated submerged Rheinwald and Green (R & G) with 5×10^{-6}M to 1×10^{-8} M retinoic acid (RA; all trans) added to normal culture medium supplemented with fetal calf serum (which already contains ~ 10^{-9} M RA). In all cases, integrin expression was confined to basal keratinocytes.

4.2.6 Suprabasal integrin transcription— *In situ* RNA hybridization

Although I could detect integrin protein suprabasally, this does not necessarily mean that integrin gene transcription is taking place suprabasally. The transcription of keratins 5 and 14 only takes place basally, but the protein is retained in the keratinocytes throughout terminal differentiation (Stoler *et al*, 1988). The turnover time of integrin protein has a half-life of about 12 hours (Hotchin and Watt 1992). If the integrins retain their normal turnover time in the wound, then this suggests that transcription is taking place suprabasally, since it takes well over 12 hours for basal cells to reach the upper suprabasal layers, even when hyperproliferative (Halprin 1972). The protein turnover time could

be lengthened, however, in the wound, allowing integrins to be retained on the cell surface. It is important to identify whether suprabasal transcription or longer protein half-life is occurring; the most definitive way to approach the question is with *in situ* RNA hybridization. Although this method would not distinguish between *de novo* transcription and increased mRNA stability, the half-lives of α_5 and β_1 mRNA in cultured keratinocytes are not altered by suspension-induced terminal differentiation (Hotchin and Watt 1992).

4.2.6.1 *In situ* hybridization

I initially followed a method from Craig Thompson, NIH, Bethesda MD. At first, I used a tritiated ribonucleotide (UTP), as the resolution is very good, although ^{3}H had the significant drawback of requiring long exposure times due to its low emission energy. I decided to use a ^{35}S nucleotide, as it offered significantly shorter exposure times and made optimization of the method much quicker.

4.2.6.1.1 Cultured cells

Both as a control, and to compare to Northern blotting results (Nicholson and Watt 1991), I used the α_6 and β_1 probes on keratinocytes cultured in either standard (Fig. 12) or low calcium medium (not shown). High levels of integrin message could be detected in all basal cells (Fig. 12), suprabasal cells were negative (seen in the center of normal calcium cultures). Basal cells appear negative in the center of stratified colonies; this is most likely due to inability of the ^{35}S β particles to reach the emulsion located several cells above the basal cells.

4.2.6.1.2 Tissue

Sections can be prepared in several ways, the main variables being fixation and embedding. Fixation in paraformaldehyde, for frozen sectioning, or in formaldehyde, for paraffin sectioning, have both been used successfully (a review of theoretical and practical considerations can

(Text continues on p. 200)

Figure 11 β_1 integrin staining (A) and morphology (B) of a keratinocyte culture 3 days after calcium-induced re-stratification.

Scale bar = 50 μm.

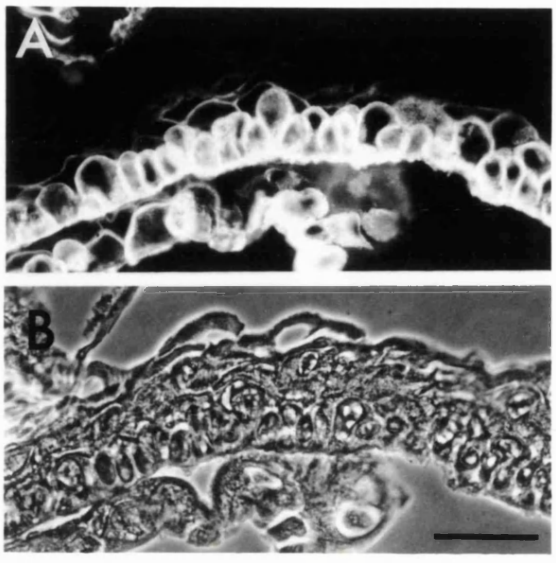

A
B

Figure 12 *In situ* hybridization of α_6 on cultured keratinocytes. A: anti-sense probe, dark field micrograph; B: anti-sense probe, phase-contrast micrograph of same field as A. C: sense control probe, dark field micrograph; D: sense control probe, phase-contrast micrograph of same field as A.

Scale bar = 50 μm.

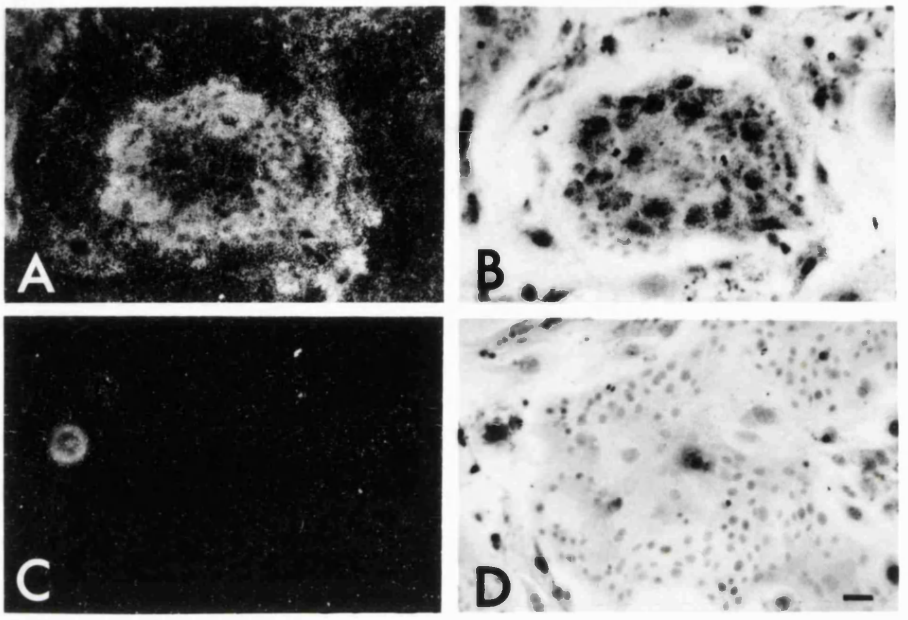

A
B
C
D

Figure 13 Phase contrast micrographs of *in situ* hybridization of α_6 and β_1 on foreskin. A: α_6 anti-sense probe; B: α_6 sense probe control; C: β_1 anti-sense probe; D: β_1 sense probe control.

Scale bar = 50 μm.

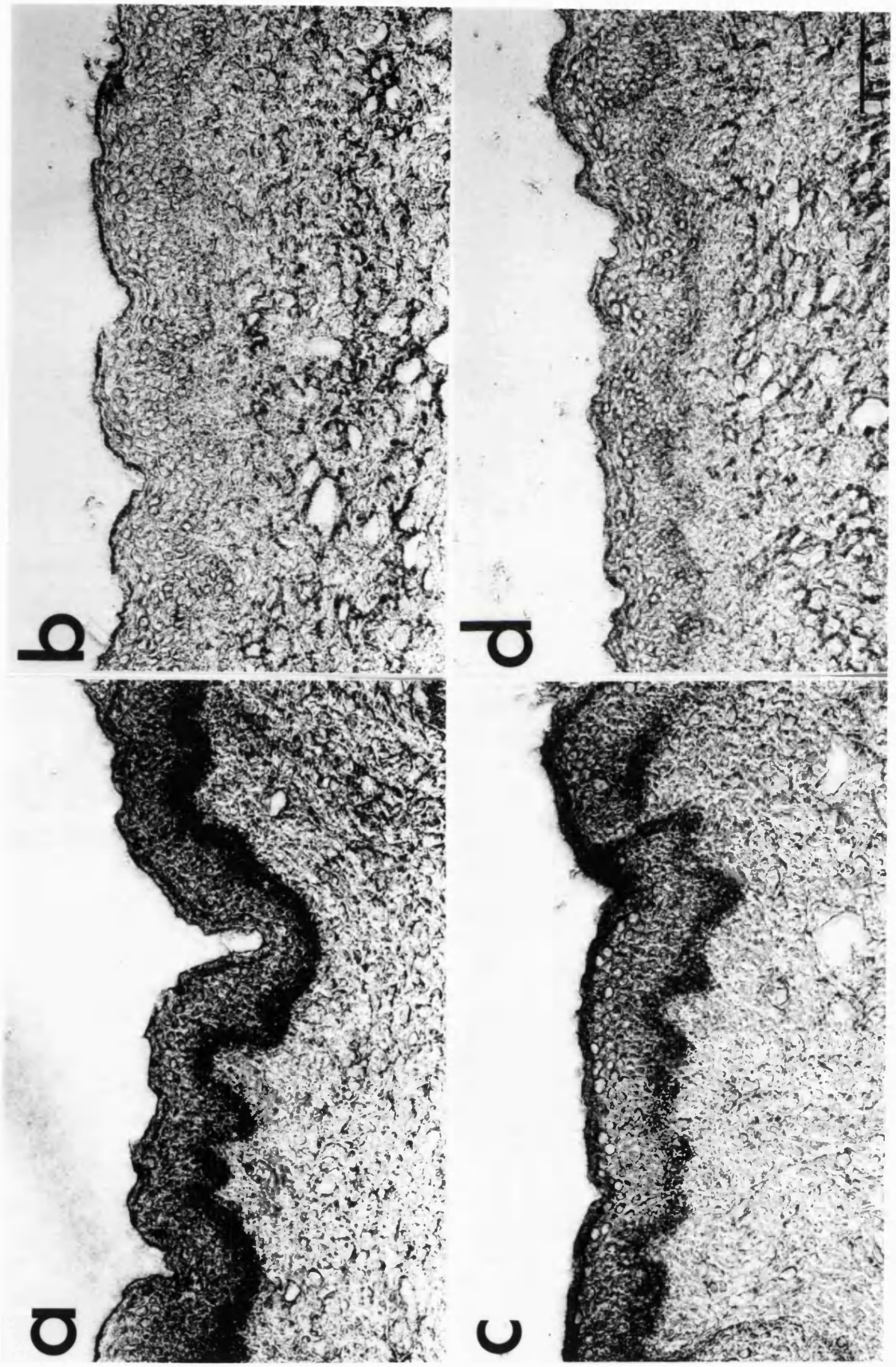
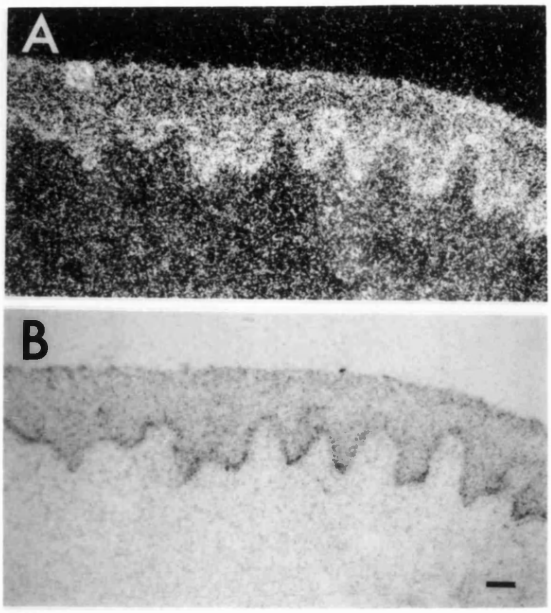

a
b
c
d

Figure 14 *In situ* hybridization of β-actin on foreskin. A: anti-sense probe, dark field micrograph; B: anti-sense probe, phase-contrast micrograph of same field as A.

Scale bar = 50 μm.


A
B

be found in (Angerer *et al*, 1987)). Frozen sections give better probe access and can therefore yield a better signal, but morphology is inferior to paraffin sections and RNA degradation can be more of a problem. I initially tried both paraffin and frozen sections of paraformaldehyde-fixed foreskin, and frozen sections of unfixed foreskin. There also can be problems with the sections coming off the slides; I tried gelatin coating or treatment with silane (Rentrop *et al*, 1986) to improve adhesion. Both gave satisfactory results, but gelatin coating had the problem of RNase contamination, which made silane treatment more attractive.

After initial technical difficulties, I was able to obtain good hybridization with both α_6 and β_1 probes to foreskin (Fig. 13), fixed before sectioning, with sections cut onto baked, silane-coated slides. All basal cells and some dermal cells were positive; suprabasal cells were negative except for background grains. The most appropriate control, hybridization under the same conditions with sense transcripts (which should have no target in the tissue) showed a slight hybridization to the basal layer of keratinocytes, but this was significantly less than the hybridization obtained with anti-sense transcripts.

Following initial success, I have been unable to duplicate these results. The most obvious potential cause was the presence of RNase, which would destroy the target and/or the probe. Skin in particular is an abundant source of RNase and diligent efforts to eliminate contamination are necessary. I attempted to eliminate RNase wherever possible, diethylpyrocarbonate treating all solutions and baking all glassware in contact with the tissue or probes. I have tried three different methods of tissue treatment and hybridization protocols, and I asked two other labs to perform the *in situ* for me with my probes. There was some limited success, but hybridization was weak and not sufficiently above background/sense controls to be definitive. Probing with β-actin as a control was successful; transcription of β-actin appeared much stronger in basal keratinocytes (Fig. 14). The ultimate aim of this work was to identify the source of the suprabasal integrin protein (i.e. basal vs. suprabasal synthesis). As with foreskin, I was unable to obtain satisfactory integrin hybridization to suction blister sections.

4.3 Discussion

The morphological changes associated with the re-epithelialization of suction blister wounds to human skin have been well documented (Kiistala and Mustakallio 1967; Hunter *et al*, 1974; Lane *et al*, 1992b). I have analyzed integrin expression during the healing process and have found a number of changes in the localization of integrin subunits compared with normal, unwounded epidermis (this work has been published (Hertle *et al*, 1992)). The most significant observation was strong suprabasal expression of several integrins at 7 days, after wound closure, in contrast to the pattern seen in normal, unwounded epidermis.

4.3.1 The blister roof

Integrins can be detected in the blister roof up until it detaches at about 3 days after wounding. Whereas in normal, attached epidermis integrins are largely confined to the basal layer, clusters of integrin-positive suprabasal cells were found in the blister roof at 24 hours. At later times, individual positively stained cells and clusters were scattered throughout the blister roof. Intracellular punctate staining with antibodies to α_6 and β_4 was observed (similar to observations of (Riddelle *et al*, 1991)), and this probably reflects internalization of hemidesmosomes and associated $\alpha_6\beta_4$ (Stepp *et al*, 1990; Sonnenberg *et al*, 1991) during detachment of the epidermis (Takahashi *et al*, 1985; Willsteed *et al*, 1991). Lane *et al* (Lane *et al*, 1992b) observed suprabasal cells in the blister roof that were expressing basal-specific keratins and suggested that basal cells are pulled upwards into a suprabasal position by the mechanical forces used to create the blister. Our observations are consistent with this proposal. Basal and suprabasal cells differ in their cohesiveness, and this may explain why basal cells were initially found as clusters (Watt 1984).

4.3.2 Lateral migration of keratinocytes

In normal epidermis keratinocyte migration is restricted to movement upwards from the basal layer, but during wound healing keratinocytes also migrate laterally. This change in behavior has been

termed "activation"; it is proposed to occur not only on wounding but also when keratinocytes are placed in culture, and it is correlated with changes in adhesiveness (reviewed in (Grinnell 1992)). Thus, when keratinocytes are first isolated from skin they adhere poorly to fibronectin, but with time in culture they develop adhesiveness to fibronectin (Takashima and Grinnell 1985; Grinnell *et al*, 1987) and also show increased migration on type I collagen and fibronectin (Guo *et al*, 1990). In contrast to normal keratinocytes, cells taken directly from a wound bed are capable of adhering to fibronectin immediately (Takashima *et al*, 1986). In wounds in which the basement membrane is destroyed, fibronectin forms the provisional matrix over which keratinocytes migrate (reviewed in (Clark 1990)) and accumulates in wound fluid, including the fluid of suction blisters (Wysocki and Grinnell 1990). Guo *et al* (Guo *et al*, 1991) have reported increased expression of the $\alpha_5\beta_1$ fibronectin receptor by keratinocytes migrating out of skin explants.

In view of such findings I was surprised to observe that during the initial stages of keratinocyte migration in suction blister wounds (until 3 days) there were no changes in integrin expression compared with normal epidermis: the same subunits were present, with the same relative staining intensity and the same cellular distribution. One possible explanation for the lack of changes is that since in suction blisters the basement membrane remains intact, the composition of the extracellular matrix over which the cells migrate may be relatively unchanged. Furthermore, it is well established that integrin function can be up- or down-regulated in the absence of changes in expression (see, for example, (Adams and Watt 1990; Du *et al*, 1991)), and so the lack of changes in staining pattern cannot be taken as evidence for unaltered integrin function. In corneal wounds, the presence or absence of a basement membrane does not significantly affect staining patterns of α_5 and α_6 (Grushkin-Lerner and Trinkaus-Randall 1991).

Hemidesmosomes assemble at the basal surface of basal keratinocytes during the lateral migration phase of wound healing, but there is some disagreement as to how far from the migrating edge they first form. There is evidence from electron microscopy (Krawczyk 1971) and staining for bullous pemphigoid antigen (a component of hemidesmosomes; (Stanley *et al*, 1981a; Robledo *et al*, 1990)) that

hemidesmosomes are formed in the leading cell. However, others have reported that they first appear several cells behind the leading cell (Odland and Ross 1968; Kurpakus *et al*, 1991; Grinnell 1992). If basement membrane components are present, hemidesmosomes are able to form (this chapter; (Krawczyk 1971; Stanley *et al*, 1981a), but if they are not (Odland and Ross 1968), hemidesmosome formation is delayed until e.g. laminin and collagen type VII are deposited by the epithelial cells (Kurpakus *et al*, 1991). The presence of ligand may therefore account for the ability to form hemidesmosomes in the leading cell. $\alpha_6\beta_4$ is not restricted to hemidesmosomes (Sonnenberg *et al*, 1991), but at the time of hemidesmosome assembly during epidermal development there is a redistribution of $\alpha_6\beta_4$ so that it becomes concentrated at the basement membrane zone (Chapter 2; (Hertle *et al*, 1991)). Kurpakus *et al* have also reported a chronological correlation between polarization of $\alpha_6\beta_4$ expression and hemidesmosome assembly in migrating corneal epithelial cells (Kurpakus *et al*, 1991). I observed a concentration of $\alpha_6\beta_4$ at the basement membrane zone right up to the migrating edge in the wounds (Fig. 3 D); although this might tend to support the conclusion that hemidesmosomes are assembled in the leading cell, further experiments will be required to resolve the issue.

4.3.3 Suprabasal integrin expression

The most striking difference between normal and healing epidermis I observed was at the time of wound closure, when several of the integrin subunits were found on the surface of suprabasal keratinocytes. Our results are broadly in agreement with those of others, who have also observed suprabasal integrin staining during healing of deep skin wounds (Clark 1990; Zambruno *et al*, 1991a) and in corneal abrasions and keratectomies (Grushkin-Lerner and Trinkaus-Randall 1991). The first subunits to show a suprabasal location were α_3 and β_1 (from 3 days), followed by α_6 (4 days), then α_2. α_5 and β_4 remained primarily confined to the basal layer and α_v was only found suprabasally in one specimen, in which the keratinocytes were disorganized. It is interesting that $\alpha_3\beta_1$ was the first integrin to be detected suprabasally, since it is also one of the earliest integrins to be expressed during epidermal development (Chapter 2; (Hertle *et al*, 1991)). The observation that α_6 was

expressed in all living suprabasal layers whereas its normal partner in keratinocytes, β_4 (Carter *et al*, 1990; De Luca *et al*, 1990; Sonnenberg *et al*, 1990a; Adams and Watt 1991), was not suggests that α_6 may form a heterodimer with β_1 in the suprabasal layers, since monomeric integrins are not found on the cell surface (Bodary *et al*, 1989; Kaufmann *et al*, 1991). A similar pattern has been reported in unwounded and ulcerated oral epithelia (Jones *et al*, 1992).

An important question that remains to be answered is whether or not the suprabasal integrins are functional. I could not detect significant levels of three of the integrin ligands, fibronectin, type IV collagen and laminin, suprabasally, but the integrins might nevertheless be functional in cell-cell adhesion (Carter *et al*, 1990; Larjava *et al*, 1990; Lampugnani *et al*, 1991). During terminal differentiation in culture, keratinocytes show a reduced adhesiveness to extracellular matrix proteins prior to overt terminal differentiation; in the case of $\alpha_5\beta_1$ down-regulation of ligand binding ability without a reduction in the amount of receptor on the cell surface has been demonstrated on commitment to terminal differentiation (Adams and Watt 1990). Since the suprabasal integrin-positive cells were expressing three markers of terminal differentiation — keratins 10 & 16, and involucrin — it is possible that the integrins, though expressed, are functionally down-regulated in the suprabasal layers. However, it is still significant that integrins are expressed concomitantly with terminal differentiation markers, an inverse relationship which is disrupted during wound healing and psoriasis.

In culture, integrin gene transcription is switched off when keratinocytes undergo terminal differentiation; the half life of the β_1 integrins under those conditions is about 12-16 hours (Hotchin and Watt 1992). Assuming that the half-life of the subunits is similar *in vivo*, this is considerably shorter than the transit time from the basal layer to the tissue surface (4-8 days in psoriasis; (Baker and Kligman 1967; Halprin 1972)). Thus, either the half-life of the proteins is longer *in vivo*, or the suprabasal, terminally differentiating keratinocytes are synthesizing integrin subunits *de novo*. Attempts at *in situ* hybridization to answer this question have been unsuccessful to date.

By the criteria of morphology, keratin 16 expression, and premature involucrin expression, the epidermis is hyperproliferative at 7 days (Weiss *et al*, 1984; Bernard *et al*, 1986; Mansbridge and Knapp 1987; Lane *et al*, 1992b) when the integrins are suprabasal, and has returned to normal by 14 days when the integrins are once more confined to the basal layer. In psoriasis, a benign hyperproliferative disorder, I noted patches of suprabasal integrin expression, as also reported by others (Kellner *et al*, 1991; Ralfkiaer *et al*, 1991). However, I did not detect the loss of integrin polarization observed by (Pellegrini *et al*, 1992) (i.e. α_6 and β_4 distribution in Fig. 10 B, C is still polarized to the BMZ). In both normal and abnormal (dysplastic and hyperplastic) oral (non- and para-keratinizing) epithelium, integrin expression can be detected suprabasally (Jones *et al*, 1992). Proliferation in normal oral epithelium is about twice that of the epidermis (Squier and Hill 1980). In healing mouth ulcers, which are hyperproliferative, integrin expression is more extensive than in normal epithelium (Jones *et al*, 1992). It seems unlikely that suprabasal integrin expression is a marker of hyperproliferation *per se*, though, since keratinocytes in culture are also hyperproliferative by these criteria (Watt *et al*, 1987) and yet in stratified cultures very little suprabasal integrin staining is observed (Fig. 11; (Adams and Watt 1991; Nicholson and Watt 1991)). It is unlikely that suprabasal integrin expression is due only to accelerated stratification, as calcium-induced rapid stratification *in vitro* did not result in suprabasal expression.

An alternative explanation is that suprabasal integrin expression is a response of keratinocytes to the inflammation characteristics of wound healing and psoriatic lesions (reviewed in (Gottlieb 1990; McKenzie and Sauder 1990b)), through which keratinocytes are exposed to a wide range of cytokines (reviewed in (McKay and Leigh 1991)). Integrin expression in other cell types is known to be upregulated by a number of cytokines and growth factors that are present in wounds (reviewed in (Nathan and Sporn 1991)); cytokine effects on keratinocytes is the subject of the next chapter.

Suprabasal expression of integrins may support the "rolling" mechanism of wound healing since suprabasal keratinocytes would need to attach to the basement membrane. Integrins would presumably be necessary and may account for the sustained expression by suprabasal

keratinocytes. This would not explain why expression extends through all suprabasal layers, though.

Although I was able to demonstrate basal expression of α_6 and β_1 integrin subunits in cultured keratinocytes (Fig. 12) and normal foreskin (Fig. 13), supporting published evidence (Nicholson and Watt 1991), I was unable to achieve satisfactory integrin hybridization in blister specimens. β-actin hybridization on foreskin indicated that suprabasal transcription was much less than basal (Fig. 14), which had been noted when attempting to use β-actin as a loading control for total keratinocyte RNA.

I made numerous attempts to recover satisfactory hybridization, changing protocols and solutions, and consulting other labs. There are several possibilities for the failure to achieve good hybridization on the blisters. The original fixation time of one hour may not have been enough for 5mm punch biopsies, although similarly-sized foreskin samples were adequately fixed within this time. I could have misidentified the orientation of the probe insert, and thus be attempting to localize transcripts with non-hybridizing sense probes. Experiments in which I used both directions of transcription as probes did not show positive hybridization with either probe, however. Skin is difficult to use for *in situ* hybridization studies due to its inherently high RNase contamination, but adequate fixation should overcome this. Whether or not the integrins are suprabasally transcribed is still an important question which remains to be answered.

4.4 Conclusions

Studies with cultured keratinocytes suggest a role for integrins in regulating cell-ECM adhesion, cell-cell adhesion, and the initiation of terminal differentiation (Adams and Watt 1990; Carter *et al*, 1990; De Luca *et al*, 1990; Larjava *et al*, 1990). During development, there are marked changes in the types of integrin subunits expressed and their location within the epidermal basal layer (Chapter 2; (Hertle *et al*, 1991)). I have demonstrated that during wound healing, and in psoriasis, integrin expression is not downregulated at the onset of terminal differentiation. Taken together, these results suggest that integrin expression is subject to

complex regulatory mechanisms, probably both environmental and developmentally programmed. The functional consequences of this dynamic regulation remain to be investigated.

Chapter 5

Cytokine modulation of integrin expression and function in keratinocytes.

5.1 Introduction

Aberrant integrin expression has been observed during wound healing and in psoriasis (Chapter 3; (Kellner *et al*, 1991; Ralfkiaer *et al*, 1991; Zambruno *et al*, 1991a; Hertle *et al*, 1992)). In wounds, the most significant observation was the immunofluorescent localization of integrins in suprabasal, terminally differentiating keratinocytes at the time of wound closure. Patches of suprabasal integrin expression were also found in involved psoriatic lesions. As described in Chapter 4, rapid stratification did not appear to cause suprabasal integrin expression, nor did hyperproliferation, since keratinocyte cultures, which are hyperproliferative by the same criteria as the healing wounds and psoriatic lesions, do not express integrins suprabasally.

Cytokines are non-immunoglobulin proteins released by cells which act at very low concentrations to regulate cell function; they are proposed to have a central role in the remodeling of tissues (Nathan and Sporn 1991). Some peptide "growth" factors (EGF, FGF, PDGF, TGF-α, TGF-β) can be characterized as cytokines (Nathan and Sporn 1991). Inflammatory cytokines (i.e. involved in stimulating inflammation) are present in both wound healing and in psoriasis, and are know to affect integrin expression.

5.1.1 Cytokine effects on integrins

The consequences of cytokine treatment on normal keratinocyte integrins have not been examined extensively, but have been studied in squamous carcinoma cells and several cell lines. The effects of IFN-γ, TGF-β, TNF-α, and IL-1β have been analyzed; both increases and decreases in integrin synthesis, depending on the cell type studied and environmental factors, have been reported. Interaction between adhesion and cytokine receptors through second messengers has been proposed (Nathan and Sporn 1991); this reinforces the likelihood that integrins will be modulated by cytokines.

Increases in integrin gene transcription have been reported after treatment with EGF, PDGF, TGF-β or IL-1β; additionally, TNF-α and IL-1β

are able to induce *de novo* α_1 subunit expression. Addition of EGF or PDGF to Swiss 3T3 cells increased β_1 integrin mRNA (Bellas *et al*, 1991). Nuclear run-on assays indicated that transcription was activated up to 7-fold within 40 minutes. This places the integrins in the group of immediate early growth-responsive genes, along with c-*fos*, c-*myc*, actin, and fibronectin (Bellas *et al*, 1991). Addition of TGF-β to 3T3-L1 preadipocytes increased both β_1 integrin mRNA and the rate of post-translational processing (Ignotz and Massagué 1987). Heino and Massagué found that α_2, α_5, and β_1 mRNA increased in MG-63, a human osteosarcoma cell line (Heino and Massagué 1989). TGF-β treatment of the WI-38 lung fibroblast line elevated α_1, α_2, α_3, α_5, and β_1 subunit mRNA (Heino *et al*, 1989). IL-1β increased the synthesis of α_1 in human skin fibroblasts (Santala and Heino 1991).

TNF-α and IL-1β apparently have the ability to induce *de novo* expression of the α_1 subunit in previously negative MG-63 cells (Santala and Heino 1991). TNF-α was also able to induce α_1 subunit expression in cultured human umbilical vein endothelial cells (HUVEC) (Defilippi *et al*, 1991b).

Only one instance of a decrease in integrin gene transcription has been reported: TGF-β treatment of MG-63 cells resulted in a decrease in α_3 mRNA (Heino and Massagué 1989).

IL-1β, TNF-α, and TGF-β have been reported to cause increases in integrin protein expression. In a melanoma cell line, treatment with IL-1β or TNF-α resulted in small increases in β_1 fluorescence (Mortarini *et al*, 1991). Heino and Massagué found that TGF-β treatment increased α_2, α_5, and β_1 protein in MG-63 (Heino and Massagué 1989). Likewise, TGF-β elevated α_1, α_2, α_3, α_5, and β_1 subunit protein on the WI-38 lung fibroblast line (Heino *et al*, 1989); the maturation rate of β_1 was also increased.

IFN-γ, TNF-α, and TGF-β can cause decreases in integrin protein expression. Treatment with IFN-γ resulted in small decreases in β_1 fluorescence in a melanoma cell line (Mortarini *et al*, 1991). TGF-β decreased α_3 protein in MG-63 (Heino and Massagué 1989). The $\alpha_v\beta_3$ vitronectin receptor on the surface of HUVEC treated with a combination

of TNF-α and IFN-γ decreased by 70%, due to translational control of β_3 (Defilippi *et al*, 1991a).

Generally, increases in integrin subunit protein result in increased adhesion to ECM substrates. Addition of TGF-β to 3T3-L1 preadipocytes increased adhesion to fibronectin and collagen type I (Ignotz and Massagué 1987). TNF-α increased HUVEC adhesion to collagen and laminin (Defilippi *et al*, 1991b). Both TNF-α and IL-1β induced an increase in MG-63 cell adhesion to laminin concomitant with the induced α_1 expression (Santala and Heino 1991).

Likewise, decreases in integrin subunit protein result in decreased adhesion. Schuger *et al* (Schuger *et al*, 1990) reported that treatment of a squamous carcinoma cell line with a combination of TNF-α and IFN-γ resulted in a decrease in adhesion (to tissue culture plastic). Heino and Massagué found that TGF-β treatment of MG-63 cells caused a decrease in laminin adhesion, but no change in adhesion to fibronectin or collagen I (Heino and Massagué 1989). HUVEC adhesion to vitronectin was reduced after a combination of TNF-α and IFN-γ treatment, while fibronectin adhesion remained the same (Defilippi *et al*, 1991a).

It is also possible that cytokines could have effects on some of the mechanisms of integrin functional modification that have been reported: through associated proteins (Brown *et al*, 1990), membrane lipid alterations (Hermanowski-Vosatka *et al*, 1992), different states of affinity (Neugebauer and Reichardt 1991), cation binding (Kirchhofer *et al*, 1991; Dransfield *et al*, 1992b), integrin glycosylation (Chammas *et al*, 1991), and possibly phosphorylation (Shattil and Brugge 1991).

There are thus several examples of cytokine effects on integrin expression, generally at the transcriptional level, but also at the post-translational level. Cytokine effects on integrins are dependent on the cell type being studied; although integrin expression in general increases, the converse also occurs. Cytokine-mediated integrin changes also have functional effects: adhesion to specific substrates is altered if integrin expression is altered. I have therefore explored the possibility that suprabasal integrin expression might be cytokine-induced.

5.1.2 Epidermal cytokines in normal skin and during inflammmation

Cytokines are involved in many aspects of intercellular communication, e.g. induction and repression of proliferation (Grossman *et al*, 1989; Choi and Fuchs 1990; Kane *et al*, 1991; Krieg *et al*, 1991), induction of migration (Barrandon and Green 1987a; Donaldson and Mahan 1988; Nickoloff *et al*, 1988), and communication between cells of the immune system (reviewed by (Nathan and Sporn 1991)). Cytokines can be classed into interleukins, interferons, colony-stimulating factors, or peptide growth factors (Nathan and Sporn 1991).

Migration *in vitro* can be stimulated by a number of cytokines, probably both through direct effects and indirect effects, such as increases in ECM synthesis (Nathan and Sporn 1991). However, the literature is contradictory on particular cytokine effects on migration. TGF-β increases migration out of an explant culture (Hebda 1988), but complications due to cytokines released by other cells of the explant make it nearly impossible to reach definitive conclusions about any one cytokine with this system. Keratinocyte migration out of an agarose drop explant, and into micropore filters in a modified Boyden chamber, is somewhat simpler. Using this model, TGF-β was found to stimulate migration, while IFN-γ inhibited migration (Nickoloff *et al*, 1988). However, this method still cannot differentiate between increased migration and increased proliferation, important since increased proliferation in itself could increase lateral migration. Sarret *et al* (Sarret *et al*, 1992) found that neither EGF, bFGF, nor TGF-β increased keratinocyte migration on plastic or collagen, when the effects on migration and proliferation were separated (method originally described in (O'Keefe *et al*, 1985; Woodley *et al*, 1988)).

The ECM is closely involved in regulating cytokine localization and function (reviewed in (Nathan and Sporn 1991; Thiery and Boyer 1992)). Matrix can bind and in some cases present cytokines to cells; basic FGF (bFGF) is the best-characterized example, in which ECM binding is essential for bFGF responses (reviewed in (Flaumenhaft and Rifkin 1991; Thiery and Boyer 1992)). It has been proposed that localized responses to circulating cytokines may be permitted by a combination of the diffusible cytokine message and non-diffusible matrix context (Nathan and Sporn

1991). It is possible that changes in the ECM during wound healing could modulate cytokine activity. Cytokines can cause matrix alteration — remodeling, secretion, and induction of proteases and their inhibitors (e.g. (Wikner *et al*, 1990)). Cytokines can also stimulate ECM expression; TGF-β is a good example. Fibronectin (Wikner *et al*, 1988) and collagen (Ladin and Morhenn 1991; Olsen *et al*, 1992) synthesis are stimulated by this cytokine, while enzymes responsible for ECM degradation are repressed (Moses *et al*, 1990).

Keratinocytes, like other cells, undergo a variety of responses to cytokines, dependent not only on the particular cytokine present, but also interactions between the cytokines (Lynch *et al*, 1989; Ansel *et al*, 1990). I will briefly review some of the many epidermal cytokines that have been proposed to play a role in wound healing and psoriasis.

One of the first events of wound healing is the release of platelet contents, including a number of cytokines, into the wound area (discussed in Chapter 4). T-lymphocytes, macrophages, and polymorphonuclear lymphocytes, among other cells, of the immune system are attracted to the wound (partly by cytokines released by the cells of the wound area), in turn releasing cytokines of their own. As the first line of defense against infection, the skin must be able to rapidly induce inflammatory cytokine release and expression to stimulate a systemic immune response (reviewed in (McKenzie and Sauder 1990b)). Cytokines, initially released by activated (e.g. by endotoxin) or damaged keratinocytes, are essential to attract cells of the immune system, which in turn secrete further cytokines. Downregulation of inflammatory cytokine release and the immune response are equally important, once healing or elimination of infection is accomplished, and again cytokines are intimately involved. Il-6, for example, has been implicated in this process; one of its functions is to induce "acute phase" proteins which attenuate the immune response (Sehgal *et al*, 1989). One of the main features of psoriasis is the chronic release of inflammatory cytokines; it is still unclear whether this is a consequence of psoriasis or a cause (reviewed by (Elder *et al*, 1990; Gottlieb 1990; McKay and Leigh 1991)). Inability to downregulate cytokine expression and response may be an important factor in diseases such as psoriasis (McKenzie and Sauder 1990a).

Besides the release of a particular cytokine, expression of a cytokine receptor can also be modulated; thus, localization of a particular cytokine cannot formally be taken as evidence that the cytokine has a target (Kupper 1990). Expression of the IL-1 receptor can be modulated: keratinocytes cultured in low calcium medium express low levels of IL-1 receptor unless induced to differentiate with TPA or increased calcium levels (Blanton *et al*, 1989). These changes in IL-1R were independent of changes in IL-1 production (Blanton *et al*, 1989). In addition, there is evidence for an IL-1 receptor antagonist in the epidermis (Gruaz-Chatellard *et al*, 1991; Haskill *et al*, 1991), adding a further layer of regulation of cytokine response.

Keratinocytes produce several inflammatory cytokines on injury or infection: interleukin-1α (IL-1α) and IL-1β (Kupper *et al*, 1986; Bell *et al*, 1987), IL-3 (Luger *et al*, 1988), IL-6 (Kirnbauer *et al*, 1989; Kupper *et al*, 1989), IL-8 (Larsen *et al*, 1989), and tumor necrosis factor-α (TNF-α) (Köck *et al*, 1990), among others. These cytokines elicit specific responses from the keratinocytes themselves (autocrine/paracrine stimulation) and the fibroblasts (e.g. by stimulating proliferation).

Transforming growth factors (TGFs)

Keratinocytes routinely produce several cytokines that regulate proliferation and differentiation, but also are involved in inflammation. TGF-α (and EGF, not produced by keratinocytes but it uses the same receptor) stimulate both proliferation and migration (Barrandon and Green 1987a). TGF-β, a multifunctional cytokine (reviewed in (Massagué *et al*, 1992)), is constitutively expressed and through its growth-inhibitory activity on keratinocytes (Coffey *et al*, 1988; Moses *et al*, 1991) may be important in maintaining a proliferative balance. Additionally, TGF-β induces matrix synthesis and may inhibit cell proliferation by stimulating synthesis of collagen I in particular: addition of collagen I to cultures can inhibit growth, while exogenous collagenase stimulates growth (Nugent and Newman 1989). TGF-β may be important for modulation of stimulatory cytokine signals during wound healing, to downregulate hyperproliferation when re-epithelialization is completed (Mansbridge and Hanawalt 1988). Although TGF-α expression is upregulated in psoriasis (Elder *et al*, 1989; Vassar and Fuchs 1991), I did not examine its

effect on keratinocyte integrins, since our normal culture conditions include EGF, which occupies the same receptor as TGF-α. Since suprabasal integrin expression is not seen in ordinary keratinocyte cultures, I did not consider TGF-α a likely candidate *in vivo*.

Interleukin-1 (IL-1)

Although there are two distinct IL-1 genes, the two protein products, α and β, appear to have identical activities and use the same receptor (reviewed in (Oppenheim *et al*, 1986)). In monocytes, IL-1 is synthesized as a 31kD precursor and processed extracellularly by secreted proteases to the active 17kD form. It is usually stored as an inactive pool within keratinocytes and has been hypothesized to be released by cell injury (Oppenheim *et al*, 1986). IL-1 plays a central role in the early induction of local and systemic inflammatory response; among its many functions are fever induction, chemotaxis and stimulation of proliferation of T-cells, B-cells, fibroblasts, neutrophils, macrophages, natural killer cells and endothelial cells ((Oppenheim *et al*, 1986) and reviewed in (McKenzie and Sauder 1990a)). Because it can be the first cytokine released, does not require a stimulus other than injury, and is not normally present in detectable quantities, IL-1 may be the primary cytokine that sets off the immunological reaction attracting leukocytes to the wound (Kupper 1990). IL-1 (probably IL-1α) has been found to accelerate wound healing (Sauder *et al*, 1990).

Extraction of IL-1 from the scraped cornified layers of volunteers and subsequent intradermal injection into the same volunteers results in an inflammatory reaction, indicating that immunoreactive IL-1 is sequestered in normal skin (Camp *et al*, 1990). The major form produced by keratinocytes is IL-1α (Camp *et al*, 1990; Mizutani *et al*, 1991); although keratinocytes can secrete pro-IL-1β, they cannot convert the pro-IL-1β form into an active form (Mizutani *et al*, 1991). It is important to note that IL-1 has no signal sequence (typically regarded essential for secretion), implying that cells do not normally secrete IL-1 (Oppenheim *et al*, 1986). When keratinocytes are grown to confluence, the level of the IL-1 receptor on the cell surface decreases (Blanton *et al*, 1989).

Interleukin-6 (IL-6)

IL-6, produced by keratinocytes *in vitro* (Kirnbauer *et al*, 1989) is an autocrine keratinocyte growth factor. Increased expression is a prominent feature of psoriasis (Grossman *et al*, 1989; Prens *et al*, 1990; Neuner *et al*, 1991) and may be important in stimulation of proliferation for wound healing (McKenzie and Sauder 1990a). IL-6 expression can be induced by IL-1 (Kupper *et al*, 1989). An important function of IL-6 is the induction of acute phase proteins in the liver, which act to down-regulate the inflammatory response (Sehgal *et al*, 1989).

Interleukin-8 (IL-8)

IL-8 (interleukin 8) has been detected in psoriasis, but is not present in normal skin (Camp *et al*, 1986). Focal expression of IL-8 mRNA has been detected by *in situ* hybridization in the upper keratinocyte layers (but not the lower or dermal) in psoriasis, suggesting that the keratinocytes are the source of IL-8 (Gillitzer *et al*, 1991). IL-8 is chemotactic for neutrophils and T-lymphocytes; its expression can be induced by IL-1 (Larsen *et al*, 1989) or TNF-α (Larsen *et al*, 1989; Barker *et al*, 1991).

Tumor necrosis factor-α (TNF-α)

Originally named for its tumor cell cytotoxicity, TNF-α has similar effects to IL-1. TNF-α helps recruit leukocytes by inducing ICAM-1 expression on endothelial cells and fibroblasts (Dustin *et al*, 1986), directing the leukocytes to the wound area. Normal cultured keratinocytes do not make TNF-α, unless stimulated with LPS (lipopolysaccharide, a bacterial product) or UV light (analogous to UVB-induced sunburn) (Köck *et al*, 1990). It has been suggested that TNF-α increases the rate of keratinocyte terminal differentiation by increasing vitamin D synthesis (Bikle *et al*, 1991). TNF-α and IFN-γ appear to interact synergistically in regulating integrin synthesis (Barker *et al*, 1990; Schuger *et al*, 1990; Defilippi *et al*, 1991a).

Interferon-γ (IFN-γ)

IFN-γ in the epidermis is usually produced by activated T-cells; its main role appears to be the induction of ICAM-1 — an intercellular adhesion molecule — and HLA-DR — an antigen-presenting molecule (Basham *et al*, 1984). ICAM-1 allows binding and activation of immigrant LFA-1-expressing T-lymphocytes (Dustin *et al*, 1988). Keratinocytes express the IFN-γ receptor constitutively (Nickoloff 1987). IFN-γ induces an anti-viral response in cells (Issacs and Lindenmann 1957). IFN-γ has generally been reported to decrease keratinocyte proliferation (Hancock *et al*, 1988; Symington 1989) although there is a preliminary report that proliferation is stimulated (Barker *et al*, 1992). Some of the confusion may be due to the differentiative effects of IFN-γ: induction of differentiation (Nickoloff *et al*, 1986) will reduce the number of proliferating cells as a proportion of the population.

Cytokines, through their multiple activities, known effects on integrin expression, and presence in both healing wounds and psoriasis, therefore seemed likely candidates for induction of suprabasal integrin expression in both wound healing and psoriasis. I restricted the cytokines for analysis to those known to have effects on integrin expression, and known to be present in both healing wounds and psoriasis. I have used several approaches to quantify cytokine effects on integrin expression and function *in vitro*, and used *in vivo* models of cytokine-induced inflammation.

5.2 Results

5.2.1 Cell surface integrin expression

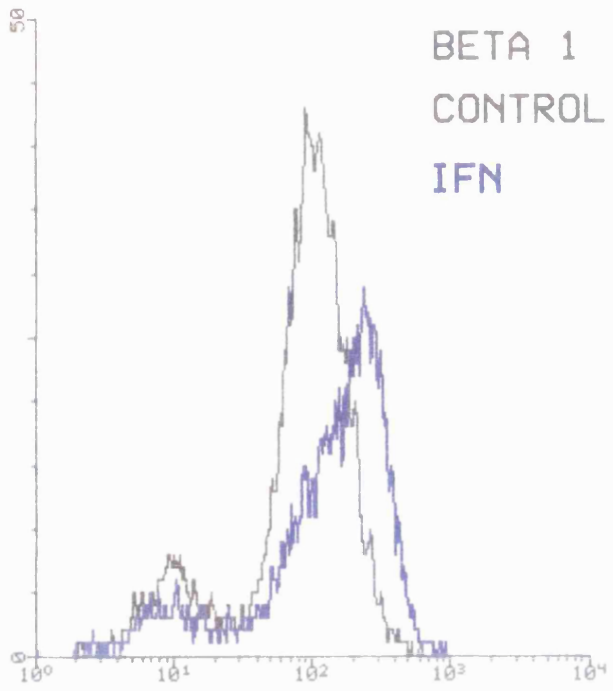
In collaboration with Phil Jones (Keratinocyte Lab), keratinocyte cultures treated with cytokines were evaluated for integrin expression by flow cytometry. Flow cytometry provides a rapid and quantitative assay of integrin levels in both basal and suprabasal keratinocytes. When a single cell suspension of keratinocytes is labelled with an anti-integrin antibody, one typically sees one major peak of brightly fluorescent cells and a smaller peak or shoulder of more weaklyfluorescent cells. Dual labeling with integrin and PNA (peanut agglutinin, a lectin expressed by terminally differentiating keratinocytes) indicates that the main peak consists of basal cells and cells with lower integrin levels are terminally differentiating (see Fig. 1). Cytokines that induce suprabasal integrin expression should cause loss of the population of low-fluorescing cells; cytokines that increase expression on basal cells should cause a shift to the right of the main peak.

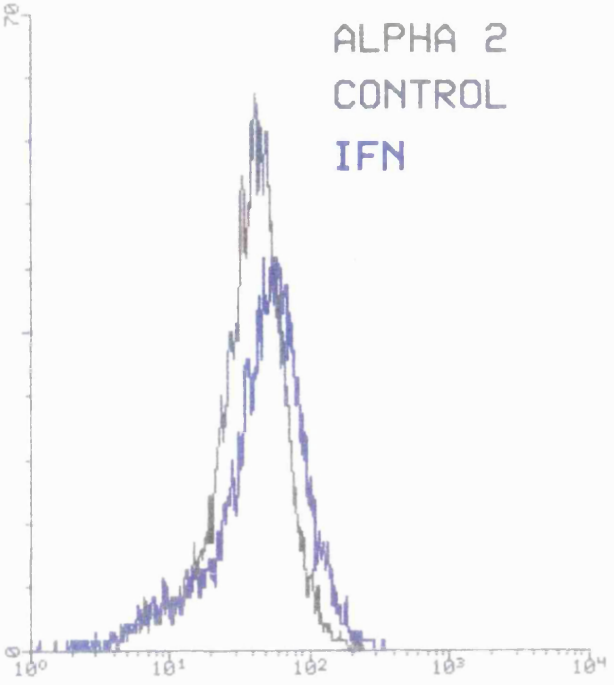
IFN-γ and TGF-β were assessed in some detail by flow cytometry, and IL-1β, IL-6, Il-8, and TNF-α were examined preliminarily. No effect on suprabasal expression could be detected (Fig. 1 and results not shown). Since suprabasal integrin expression did not appear to be cytokine inducible, by flow cytometric analysis, I decided to concentrate on changes that might be occurring in the basal cells. In order to evaluate changes in the basal cells, the modal fluorescence value (the channel number for the highest point of the major peak on the histogram) was used, as it would reflect changes occurring in the majority of the cells.

Although in some experiments there was an increase in integrin expression (e.g. Fig. 1), subsequent experiments revealed considerable variation in the level of integrin expression after cytokine treatment. To test the effects of time and culture conditions, keratinocytes were treated for 24 or 48 hours, and in normal (10% fetal calf serum and HICE (Chapter 2)) or low serum (0.5% fetal calf serum and no added growth factors) medium.

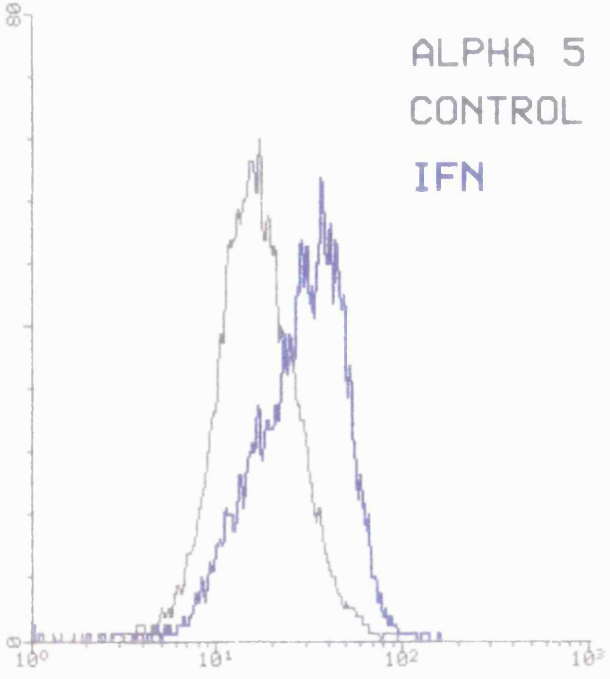
(Text continues on p. 227)

Figure 1 Representative flow cytometric histograms of control (black profile) and 500U/ml IFN-γ-treated (blue profile) keratinocytes, stained with the integrin antibody indicated in each panel. IFN-γ treatment was for 48 hours in normal medium. In this experiment, there was an increase relative to control, as indicated by the increased fluorescence intensity of the main peak on treatment. The X-axis represents arbitrary fluorescence units on a log scale; the Y-axis represents the number of events (on an arbitrary scale). The small peak left of the main peak in the β_1 panel represents autofluorescence of mouse fibroblast contamination; the antibody is human-specific.





U3:PJ0203005\FL1\Fluorescence 1



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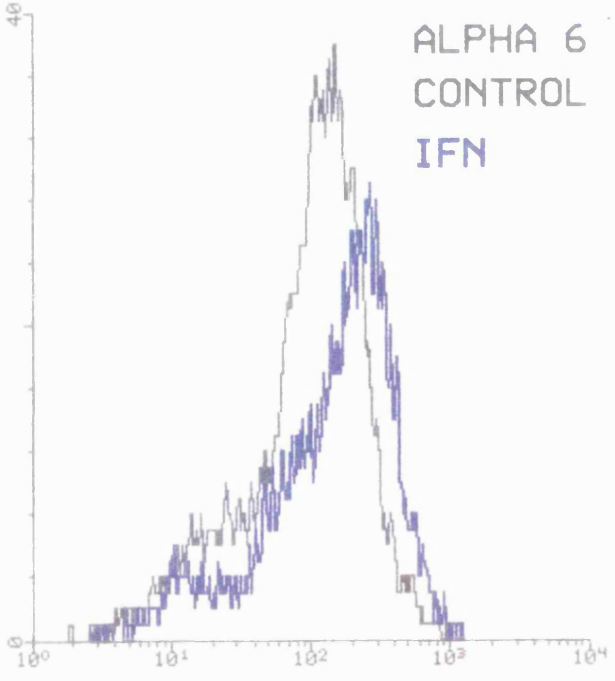


Figure 2 Flow cytometric analysis of integrin subunit expression after 24- or 48-hour treatment with 200U/ml IFN-γ. "Low serum" is FAD medium with 0.5% fetal calf serum, but no exogenous growth factors added. "Normal medium" is FAD with 10% fetal calf serum and growth supplements. Values shown are normalized percentage changes on cytokine treatment when compared to untreated controls. Each point represents the modal fluorescence value normalized against control for one experiment.

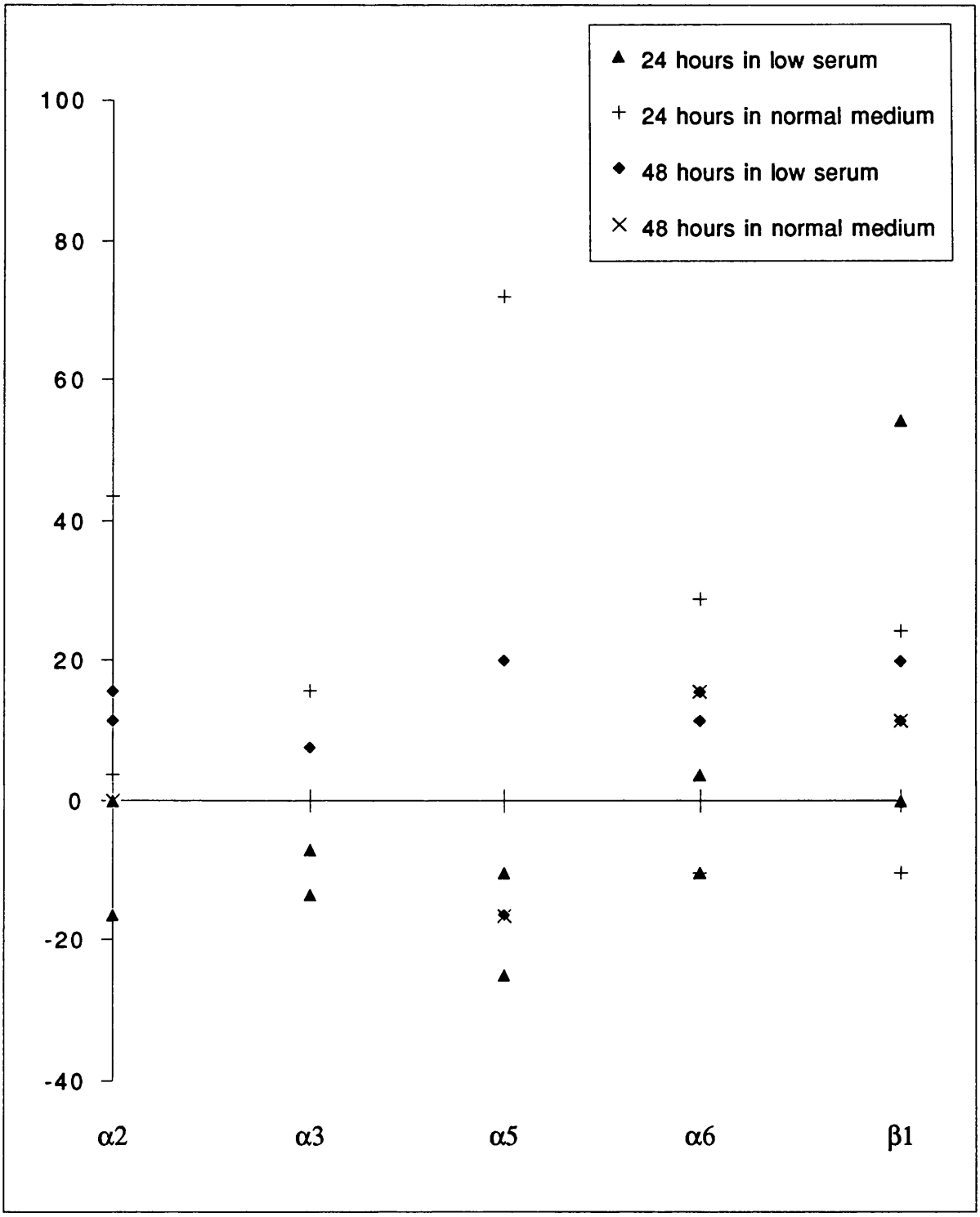

24 hours in low serum
24 hours in normal medium
48 hours in low serum
48 hours in normal medium
100
80
60
40
20
0
-20
-40
α2
α3
α5
α6
β1

Figure 3 Flow cytometric analysis of integrin subunit expression after 24- or 48-hour treatment with 500U/ml IFN-γ. "Low serum" is FAD medium with 0.5% fetal calf serum, but no exogenous growth factors added. "Normal medium" is FAD with 10% fetal calf serum and growth supplements. Values shown are normalized percentage changes on cytokine treatment when compared to untreated controls. Each point represents the modal fluorescence value normalized against control for one experiment.

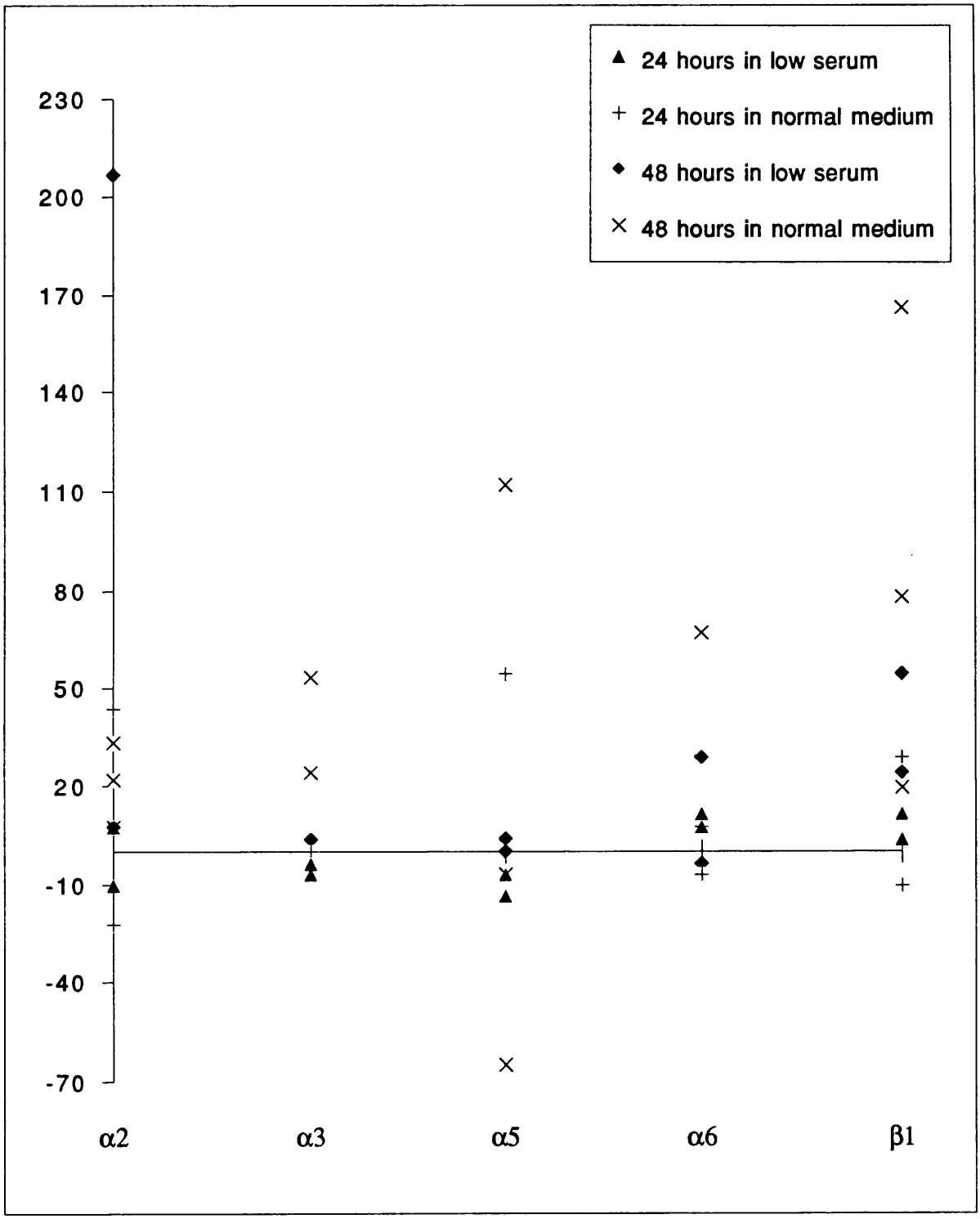
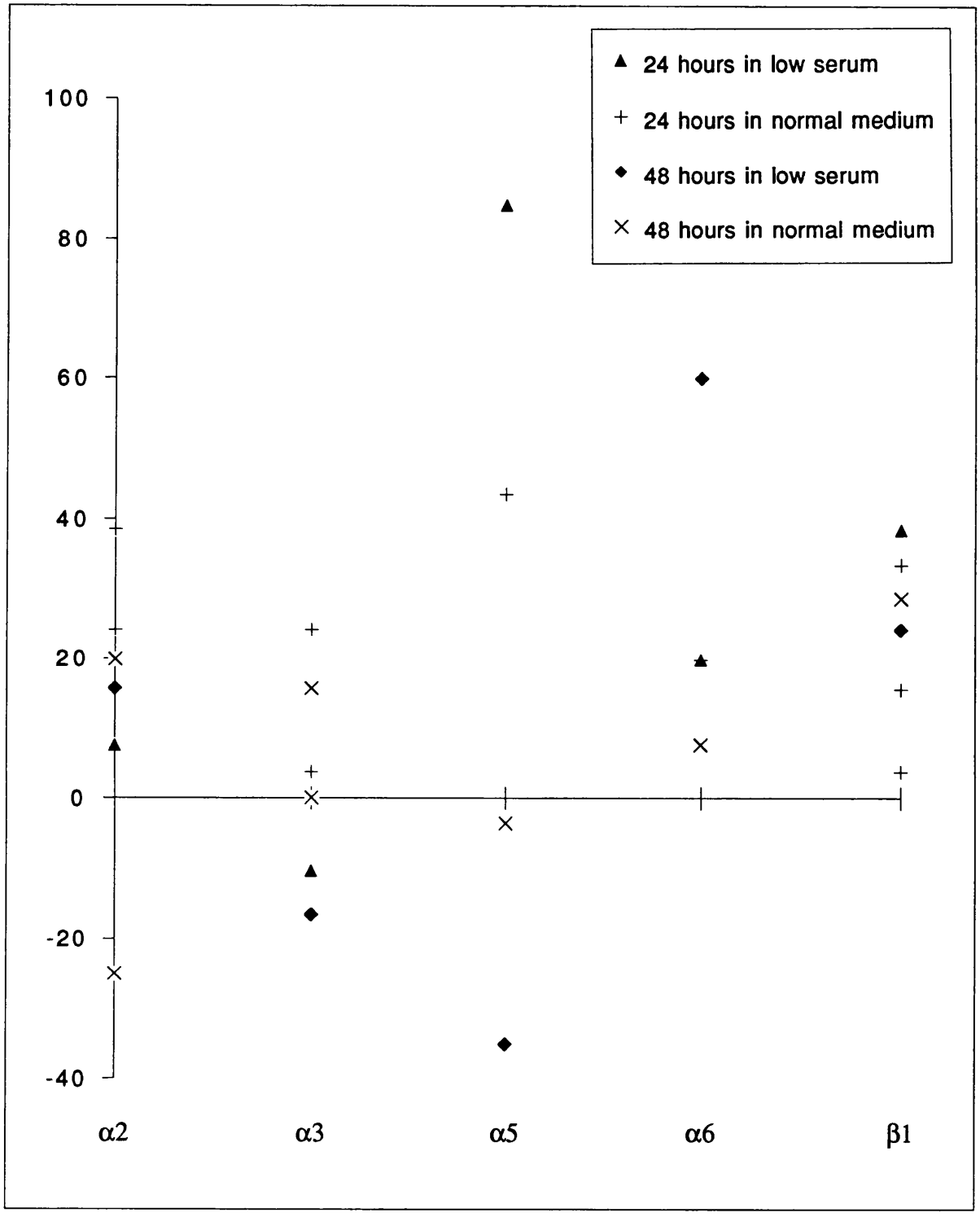

24 hours in low serum
24 hours in normal medium
48 hours in low serum
48 hours in normal medium
230
200
170
140
110
80
50
20
-10
-40
-70
α2
α3
α5
α6
β1

Figure 4 Flow cytometric analysis of integrin subunit expression after 24- or 48-hour treatment with 10ng/ml TGF-β. "Low serum" is FAD medium with 0.5% fetal calf serum, but no exogenous growth factors added. "Normal medium" is FAD with 10% fetal calf serum and growth supplements. Values shown are normalized percentage changes on cytokine treatment when compared to untreated controls. Each point represents the modal fluorescence value normalized against control for one experiment.


24 hours in low serum
24 hours in normal medium
48 hours in low serum
48 hours in normal medium
100
80
60
40
20
0
-20
-40
α2
α3
α5
α6
β1

Treatment with 200U/ml IFN-γ for 24 or 48 hours, in low serum or normal medium, is shown in Fig. 2. The data are presented as modal values — therefore representing the main peak — on a per experiment basis. 24 hour treatment in low serum medium resulted in a small decline or no change compared to control values. 24-hour treatment in normal medium resulted in increases in α_2, α_3, and α_5 fluorescence, while α_6 and β_1 values either declined or increased. 48 hour treatment in low serum medium resulted in small increases for all subunits except α_5, which declined in one experiment and increased in another. 48 hour treatment in normal medium had no consistent effects on the subunits, with both increases and decreases being observed.

The effect of treatment with 500U/ml IFN-γ for 24 or 48 hours, in low serum or normal medium, is shown in Fig. 3. 24 hour treatment in low serum medium resulted in very small increases or decreases in modal integrin fluorescence of ~10%. 24-hour treatment in normal medium resulted in both increases and decreases in α_2, α_6, and β_1. A single α_5-stained sample showed an increase of ~50%. 48 hour treatment in low serum medium resulted in small changes from control, except for an increase of 20-50% in β_1 fluorescence. 48 hour treatment in normal medium resulted in increases in α_2, α_3, and β_1 fluorescence. The level of α_6 examined in a single experiment increased by ~60% compared to control. α_5 fluorescence showed a large increase or decrease, depending on the experiment.

Treatment with 10ng/ml TGF-β for 24 or 48 hours, in low serum or normal medium, is shown in Fig. 4. 24 hour treatment in low serum medium showed increases in all subunits except for α_3. 24 hour treatment in normal medium resulted in increases in all subunits, in the range of 5-40%. 48 hour treatment in low serum medium resulted in increases in α_2, α_6, and β_1, while α_3 and α_5 decreased. 48 hour treatment in normal medium had equivocal effects, with both increases and decreases observed.

By gating on several parameters, it is possible to separate basal from suprabasal keratinocytes (see Fig. 1 in Chapter 2). A comparison of integrin expression between total (i.e. basal + suprabasal) and basal only populations was examined, but no significant difference could be observed (not shown).

The flow cytometry results suggested that there was no suprabasal integrin expression; I wanted to confirm this by examining the localization in cultured keratinocytes by immunofluorescence. Treatment for 1-7 days with 100-600U/ml IFN-γ did not induce suprabasal expression, consistent with the flow cytometry results. ICAM-1 was detected on keratinocytes treated with at least 200U/ml of IFN-γ for at least one day (not shown). ICAM-1 expression was variable, but stronger at higher IFN-γ concentrations, and was expressed by all keratinocytes, though more strongly on basal cells (not shown).

5.2.2 Integrin protein level and processing modifications

The flow cytometry data showed no effect of IFN-γ or TGF-β on suprabasal integrin expression and only small, variable effects on basal integrin expression. To examine whether these, or other, cytokines affected protein synthesis or post-translational modification of total β_1 integrins, I immunoprecipitated integrins from keratinocytes. Keratinocyte cultures were metabolically labelled overnight with ^{35}S-methionine in the presence of cytokines, then lysed and immunoprecipitated with a monoclonal anti-β_1 antibody, which specifically immunoprecipitates β_1 and associated α subunits (CD29; see also (Hotchin and Watt 1992)).

Immunoprecipitation with an anti-β_1 antibody results in three labeled bands when resolved by electrophoresis (Fig 5A and B). The top band consists of mature (processed) α subunits co-precipitated with β_1; the middle band is mature β_1; and the lower band is immature (not post-translationally modified e.g. non-glycosylated) β_1 integrin subunit. Immature α subunits are obscured by the mature β_1 band. As shown in Fig. 5, the total amount of integrins did not differ significantly between control and keratinocytes treated for 24 hours, both when immunoprecipitated on the basis of equal protein (Fig. 5A) and equal counts (Fig. 5B). Treatment for 48 hours likewise did not indicate any increase in integrin protein synthesis on cytokine treatment (not shown). Additionally, no differences in mobility of the bands (Fig. 6) were

Figure 5 β_1 integrin immunoprecipitation of metabolically labeled keratinocytes, separated by non-reducing gel electrophoresis. (A) immunoprecipitation on the basis of equal protein; and (B) immunoprecipitation on the basis of equal counts (radioactivity). Mature α, immature β, and mature β subunits are indicated. Keratinocytes were treated with cytokine, where appropriate, for 24 hours. The apparent mobility shift in the 100U/ml IL-1β lane on panel B is an artifact due to a damaged gel.

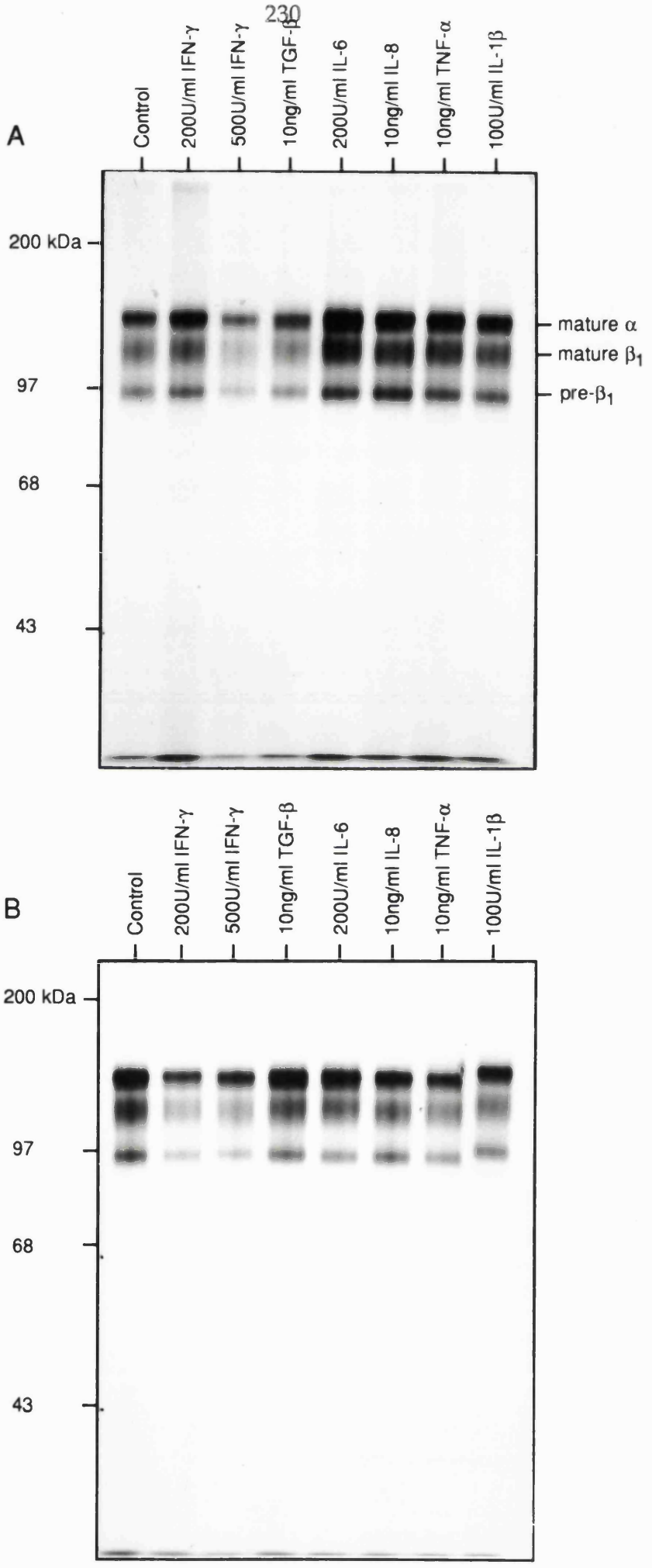
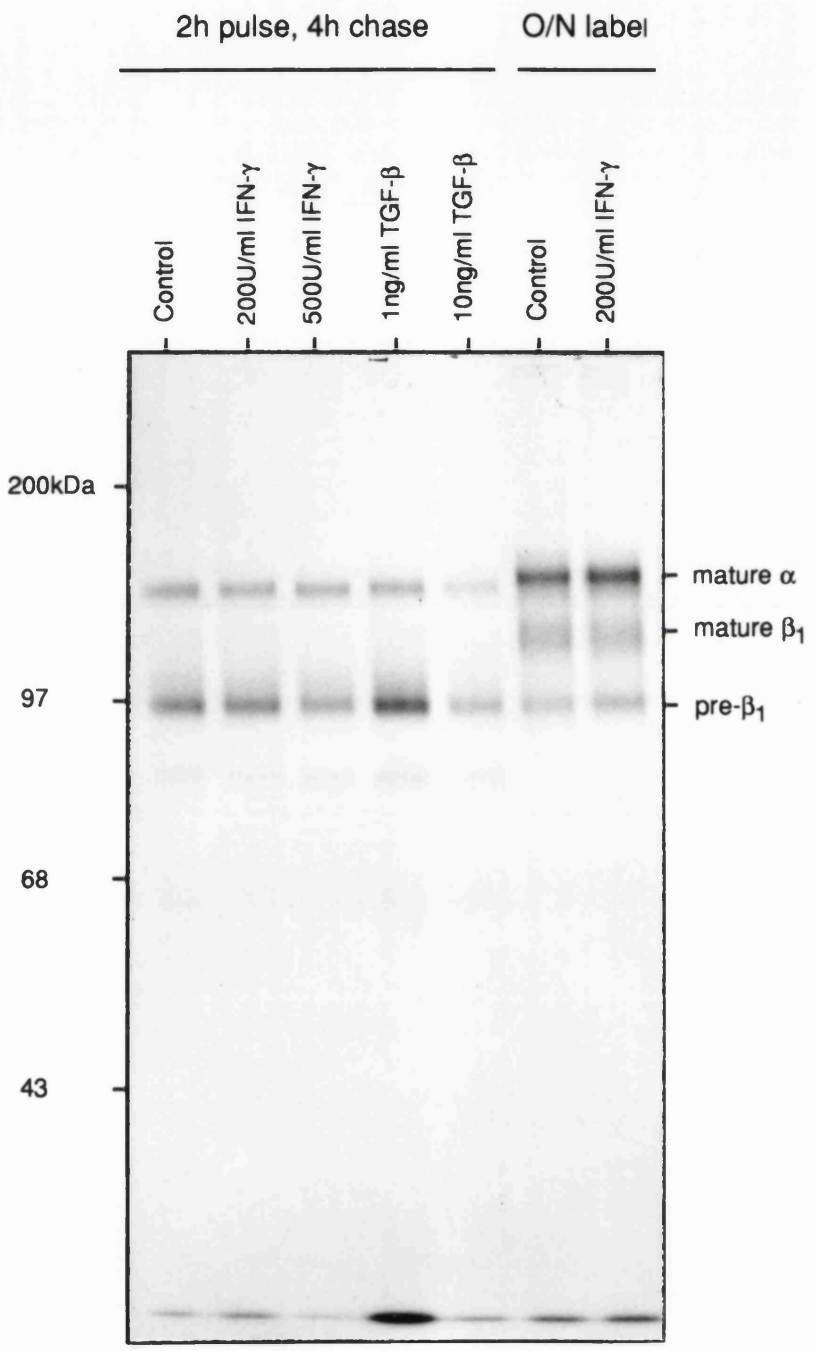

A
Control
200U/ml IFN-γ
500U/ml IFN-γ
10ng/ml TGF-β
200U/ml IL-6
10ng/ml IL-8
10ng/ml TNF-α
100U/ml IL-1β
200 kDa
97
68
43
mature α
mature β1
pre-β1
B
Control
200U/ml IFN-γ
500U/ml IFN-γ
10ng/ml TGF-β
200U/ml IL-6
10ng/ml IL-8
10ng/ml TNF-α
100U/ml IL-1β
200 kDa
97
68
43

Figure 6 β_1 immunoprecipitation of pulse-chase labeled keratinocytes, with unchased keratinocytes ("O/N label") for comparison. Keratinocytes were treated with cytokine, where indicated, for 24 hours. Note that the pulse-chase time was insufficient to generate mature integrins (Hotchin and Watt 1992); mature integrin is only apparent in the overnight labeled keratinocytes. Precipitation was on the basis of equal protein.


2h pulse, 4h chase
O/N label
Control
200U/ml IFN-γ
500U/ml IFN-γ
1ng/ml TGF-β
10ng/ml TGF-β
Control
200U/ml IFN-γ
200kDa
97
68
43
mature α
mature β1
pre-β1

observed, indicating that there were no changes in glycosylation or other post-translational modifications after cytokine treatment.

5.2.3 Integrin mRNA levels

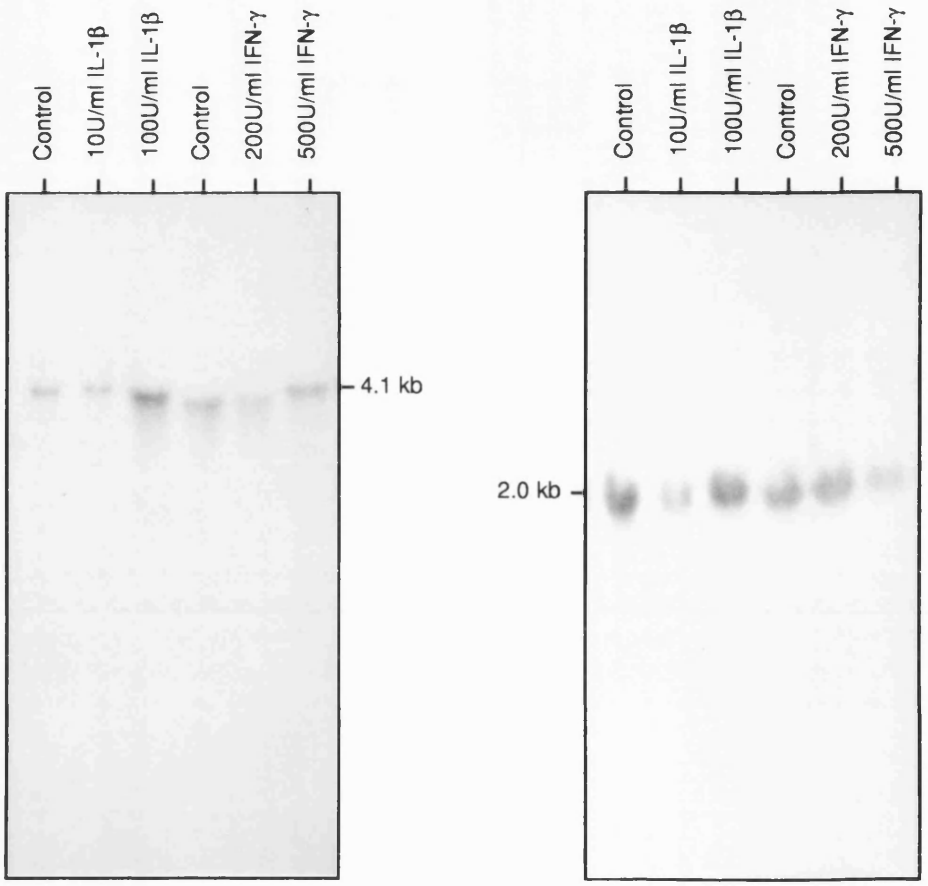
I examined the levels of β_1 mRNA in cytokine-treated keratinocytes by Northern hybridization. The β_1 probe hybridized to a band corresponding to approximately 4.1kb, the expected size (Argraves *et al*, 1987); the 18S probe, used as a loading control, hybridized to approximately 1.9kb (Sambrook *et al*, 1989). Autoradiograms of β_1 and 18S hybridization on cytokine-treated keratinocyte RNA are shown in Fig. 7. The level of integrin expression was quantified by correcting for loading errors on comparison with 18S hybridization intensity. This allowed the differences from control to be quantitated and indicated that neither IFN-γ and IL-1β induced major changes in steady-state β_1 mRNA.

The expression of ICAM-1 mRNA was also evaluated in IFN-γ and TGF-β treated keratinocytes. IFN-γ treatment induces ICAM-1 expression (Dustin *et al*, 1986; Caughman *et al*, 1990) whereas TGF-β has not been reported to. Treatment for 24 hours with IFN-γ showed a concentration-dependent induction of ICAM-1 mRNA, whereas ICAM-1 could not be detected in untreated or TGF-β-treated keratinocytes, nor in untreated J2 feeder cells (Fig. 8).

5.2.4 Cytokine effects on keratinocyte proliferation and differentiation

Most of the cytokines I used are known to have effects on the differentiation of keratinocytes; in general, the number of cells in the differentiated compartment is increased (see Introduction). I assessed the effect of cytokines on differentiation using expression of involucrin, a marker of terminal differentiation (Rice and Green 1977) expressed by all suprabasal cells in culture (Watt and Green 1982; Watt 1983). Cell suspensions of control and cytokine-treated cells were stained with an anti-involucrin antibody and the number of positive cells was determined by immunofluorescence.

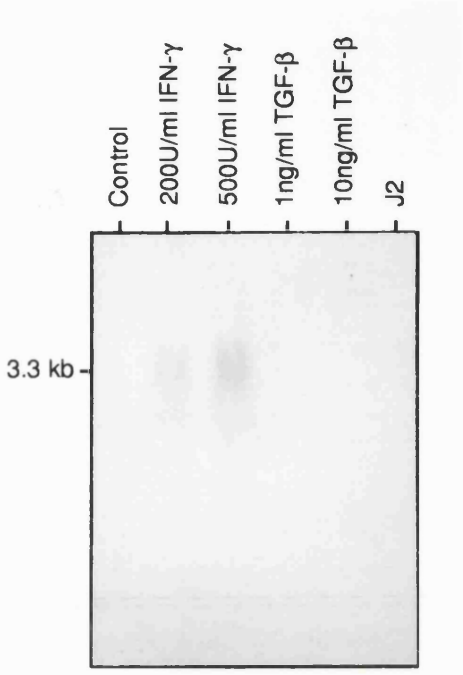
Figure 7 β_1 integrin (left panel) and 18S (right panel) RNA analysis in IL-1β and IFN-γ treated keratinocytes by Northern blotting. pFNRβ (see Chapter 5) was used as the probe for β_1. 18S RNA was used as a loading control to generate the results in Table 1. 20ug of total RNA was loaded per lane, from keratinocytes treated for 48 hours.


Control
10U/ml IL-1β
100U/ml IL-1β
Control
200U/ml IFN-γ
500U/ml IFN-γ
4.1 kb
Control
10U/ml IL-1β
100U/ml IL-1β
Control
200U/ml IFN-γ
500U/ml IFN-γ
2.0 kb

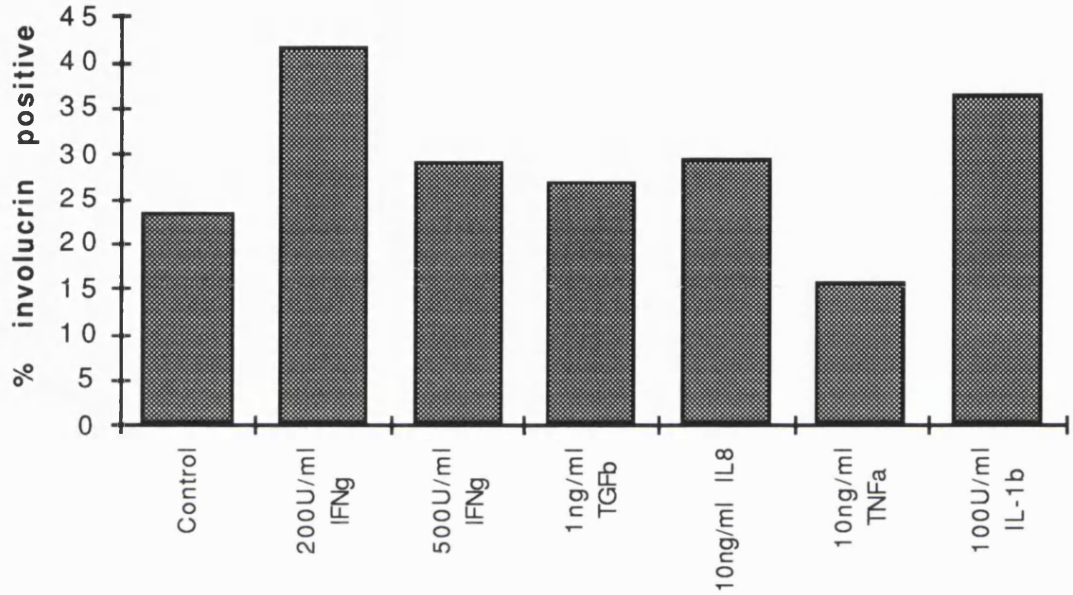
β1

18S

Figure 8 ICAM-1 RNA expression in control, IFN-γ, or TGF-β-treated keratinocytes, and in untreated J2 feeder cells. Keratinocytes were treated for 24 hours. RNA is somewhat degraded, indicated by smearing of the hybridization signal.


Control
200U/ml IFN-γ
500U/ml IFN-γ
1ng/ml TGF-β
10ng/ml TGF-β
J2
3.3 kb

The absolute percentages of involucrin-positive cells varied between experiments, but the trends were consistent. A representative result is shown below. Only TNF-α treatment reduced the number of involucrin positive cells compared to the control; the other cytokines increased the number of involucrin-positive cells.



5.2.5 Keratinocyte adhesion

Integrin function can be regulated independently of expression (Hynes 1992). This has been demonstrated in keratinocytes: 5 hours after placing cultured keratinocytes into suspension, the ability of $\alpha_5\beta_1$ to bind to fibronectin decreases, while the level of $\alpha_5\beta_1$ on the cell surface does not change (Adams and Watt 1990).

Functional regulation was tested by quantitating adhesion of cytokine-treated keratinocytes to laminin, collagen type IV, and fibronectin. Keratinocytes were treated with cytokines and allowed to adhere to 10 or 30 μg of purified ECM protein coated onto a 96-well plate well. 10μg is a suboptimal concentration and 30μg allows near-maximal adhesion (Adams and Watt 1990).

I tested three different methods for determining the number of attached cells: visual scoring of methylene-blue stained cells, measurement of hexosaminidase activity, and ^{51}chromium labelling. I found that quantifying adhesion by hexosaminidase activity, though accurate for untreated keratinocytes, was confounded by the effects of cytokines on hexosaminidase activity. For equal numbers of cells in suspension, hexosaminidase activity as judged by optical density value was different for control and cytokine-treated cells: cytokines had an effect on the enzyme which was independent of cell number (see standard curves in Chapter 2, Section 2.4.1). It was possible to correct for this, but the degree of variation between different experiments was unacceptably high (see Fig. 9). Likewise, quantifying adhesion by ^{51}Cr uptake, dependent primarily on cell size, also was affected by cytokine treatment (not shown but see Chapter 2, 2.4.2), probably because of the effects of cytokines on terminal differentiation. The most consistent results were obtained by staining adherent cells with methylene blue and counting representative fields by phase microscopy (Fig. 10).

There appeared to be no significant effect of the cytokines tested (IFN-γ, TGF-β, IL-1β, IL6, IL-8, or TNF-α) on the proportion of keratinocytes that adhere to ECM proteins. A confounding factor is the induction of differentiation that occurs on cytokine treatment. Thus, in the absence of any specific effect of cytokines on integrins, cell adhesion as a proportion of total cells would be expected to decrease on cytokine treatment, as the basal cells (i.e. those cells capable of adhering) decrease proportionally. Since there do not appear to be any decreases in adhesion on cytokine treatment, it is therefore possible that there is a slight actual increase in adhesion on cytokine treatment which is masked by the increased differentiation. In the case of TNF-α, there might be a decrease in adhesion, suggested by the lower number of involucrin positive cells (above).

Keratinocytes can synthesize and adhere to their own matrix molecules (Carter *et al*, 1991). TGF-β in particular stimulates synthesis of ECM proteins in a range of cell types (Nickoloff *et al*, 1988; Clark 1989; Amento and Beck 1991; Ladin and Morhenn 1991; Olsen *et al*, 1992); this could potentially affect the adhesion results. Addition of 20μg/ml cycloheximide (to inhibit protein synthesis during the adhesion assay)

(Text continues on p. 246)

Figure 9 Adhesion of cytokine-treated keratinocytes to 10μg of purified ECM, scored by hexosaminidase activity. Cytokine used is indicated at bottom of graph. Control adhesion is normalized to 100%. All values are expressed as percentages of control (untreated) keratinocte adhesion, after subtraction of non-specific adhesion to BSA-coated wells. Error bars are standard error of the mean.

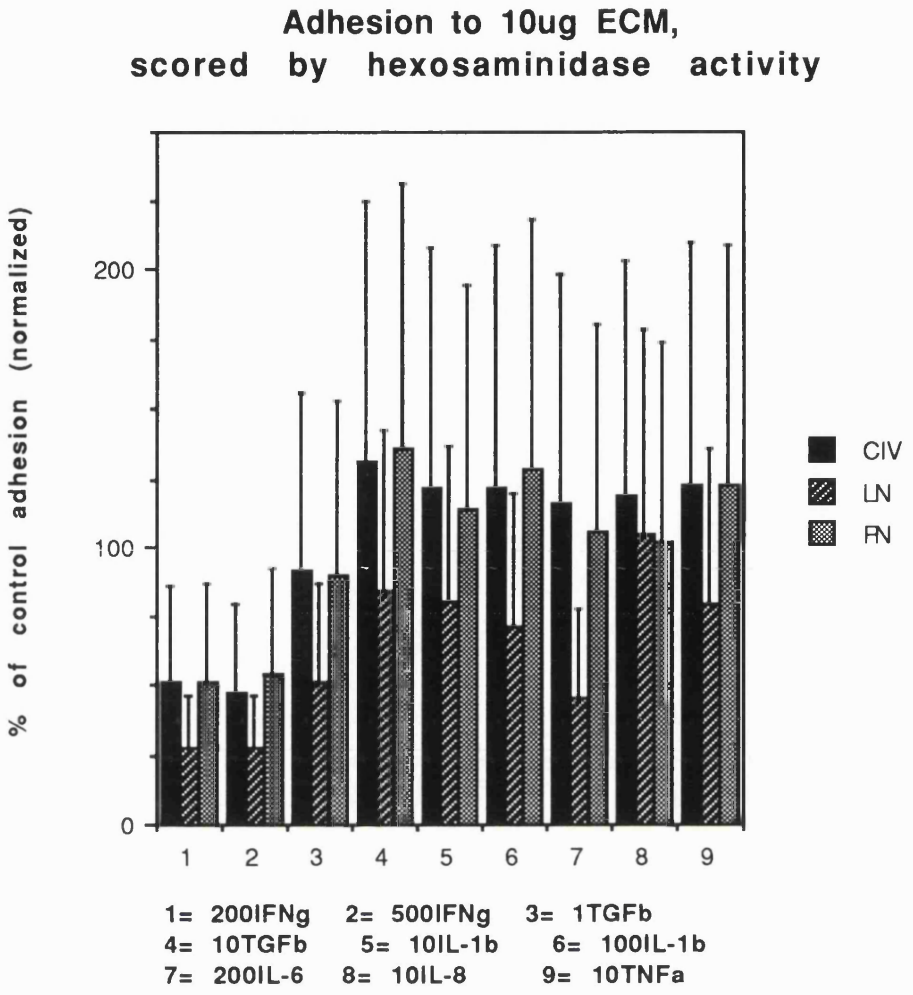

Adhesion to 10ug ECM,
scored by hexosaminidase activity
% of control adhesion (normalized)
200
100
0
1 2 3 4 5 6 7 8 9
CIV
LN
FN
1= 200IFNg 2= 500IFNg 3= 1TGFb
4= 10TGFb 5= 10IL-1b 6= 100IL-1b
7= 200IL-6 8= 10IL-8 9= 10TNFa

Figure 10 Adhesion of cytokine-treated keratinocytes to 10μg of purified ECM, scored by visual counting. Cytokine used is indicated at bottom of graph. Control adhesion is normalized to 100%. All values are expressed as percentages of control (untreated) keratinocte adhesion, after subtraction of non-specific adhesion to BSA-coated wells. Error bars are standard error of the mean. Where no error bar is shown, experiment was conducted once.

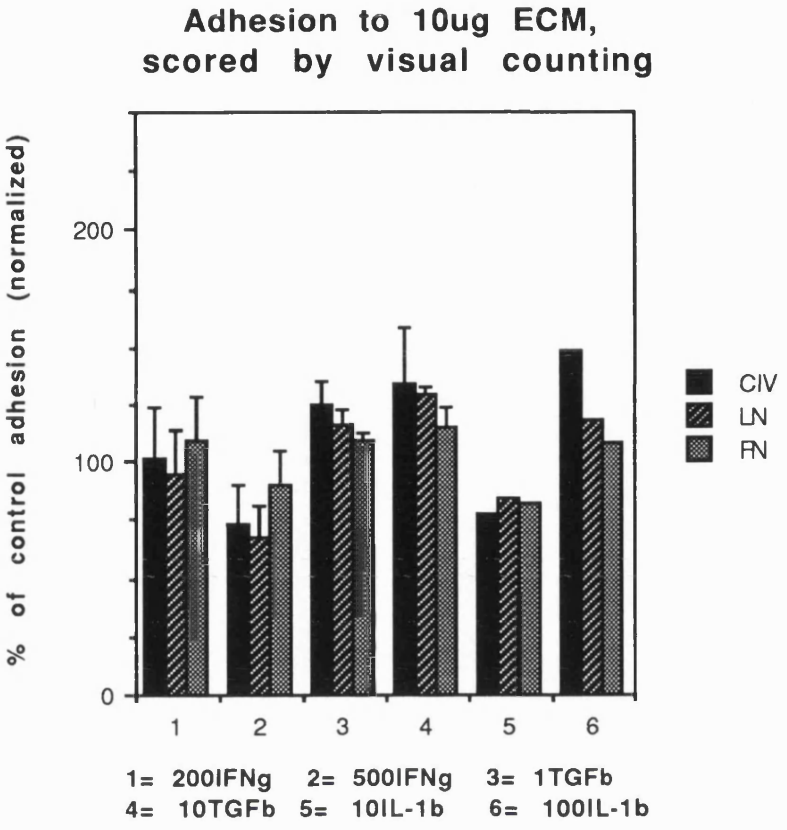
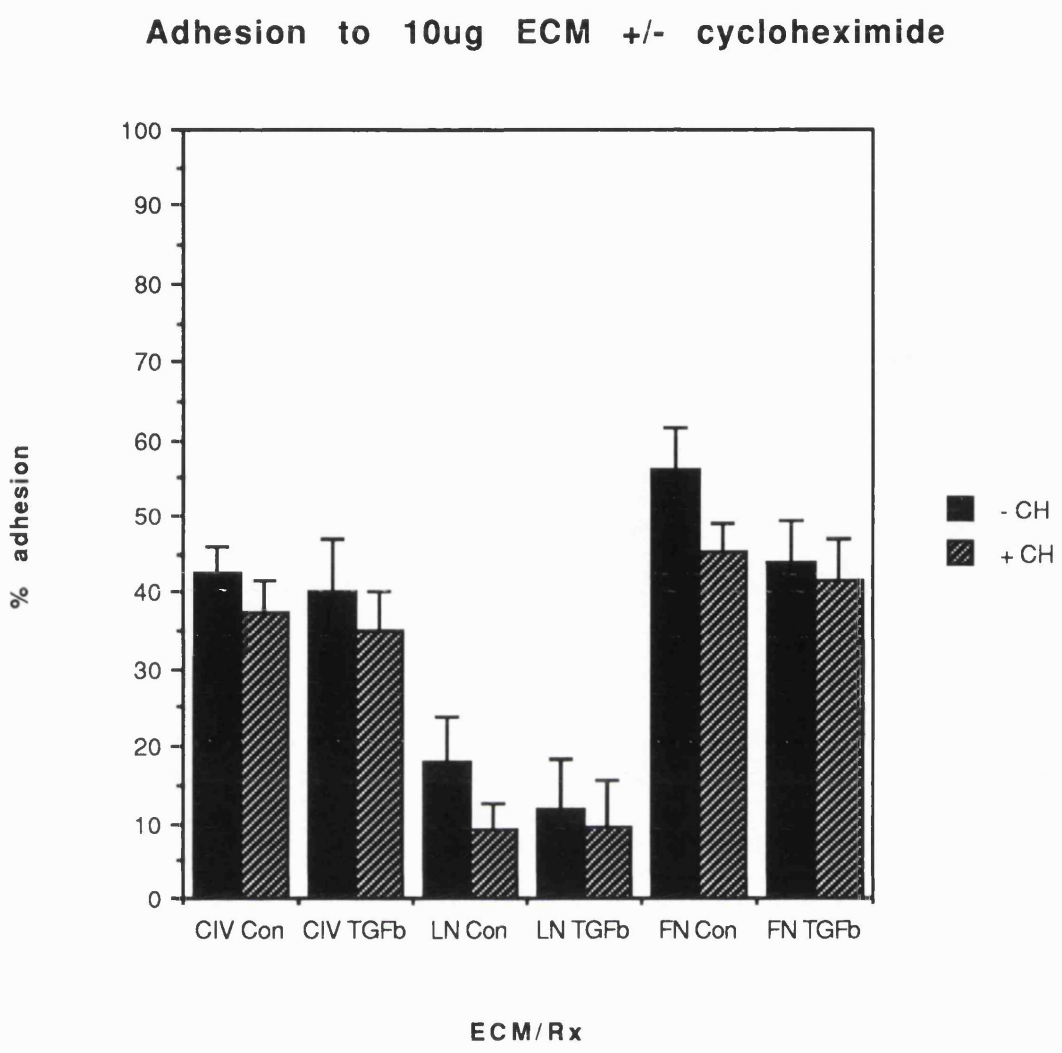

Adhesion to 10ug ECM,
scored by visual counting
% of control adhesion (normalized)
200
100
0
1
2
3
4
5
6
CIV
LN
FN
1= 200IFNg 2= 500IFNg 3= 1TGFb
4= 10TGFb 5= 10IL-1b 6= 100IL-1b

Figure 11 Adhesion of control and cytokine-treated keratinocytes to 10ug of purified ECM in the absence and presence of cycloheximide. Results are expressed as the percentage of cells added that adhere to the well. "-CH" is without added cycloheximide; "+CH" is with cycloheximide added during adhesion at 20μM. "CIV con" is control cell adhesion to collagen type IV; "CIV TGFb" is cells treated with 10ng/ml TGF-β for 24 hours, adhering to collagen type IV. Results were quantified by hexosaminidase activity. Adhesion to BSA-coated wells has been subtracted and values are corrected for cytokine effects on hexosaminidase activity. Error bars are SEM values for triplicate wells.


Adhesion to 10ug ECM +/- cycloheximide
% adhesion
100
90
80
70
60
50
40
30
20
10
0
CIV Con
CIV TGFb
LN Con
LN TGFb
FN Con
FN TGFb
ECM/Rx
- CH
+ CH

lowered the percentage of cells adhering but did not affect relative adhesion, when control and TGF-β-treated cell adhesion was compared on different ECM proteins (Fig. 11).

5.2.6 Epidermal integrin expression pattern in cytokine-injected volunteers

The overall conclusion from the *in vitro* experiments was that none of the cytokines tested had any major effects on keratinocyte integrin expression. *In vivo*, however, keratinocytes are exposed to more than one cytokine simultaneously, so the *in vitro* results do not completely rule out a role of cytokines in inducing suprabasal integrin expression. Fortunately, through Drs. Groves (Dept of Dermatology, Guy's Hospital, London) and Camp (Dermatology Section, Univ. of Leicester), I was able to obtain tissue sections from volunteers who had received intradermal injections of interferon-γ, IL-1α, or TNF-α (original work described in (Camp *et al*, 1990; Barker *et al*, 1992; Groves *et al*, 1992), triggering a classic inflammatory response with induction of markers of hyperproliferation.

Sections of skin taken from the injection site at time points ranging from 4 hours to five days were stained with antibodies against integrins and ICAM-1 (Figs. 12-14). Integrin staining for all subunits examined (α_2, α_3, α_5, α_6, β_1, and β_4) was indistinguishable from control tissue (PBS-injected; Fig. 13B), i.e. restricted to the basal layer of keratinocytes. ICAM-1 was expressed by keratinocytes in both IFN-γ and TNF-α-injected volunteers (Fig. 14), indicating that the keratinocytes had responded to the cytokines. The morphology of the epidermis was normal; an inflammatory infiltrate was present (Larsen *et al*, 1989; Groves *et al*, 1991).

5.2.7 Raft integrin expression pattern

Although I was unable to induce suprabasal integrin expression in plastic-attached cultured keratinocytes (above), I was able to recreate the pattern of suprabasal integrin expression *in vitro* with a different technique: keratinocyte raft cultures. In raft cultures, the keratinocytes are grown on collagen I gel containing feeder cells, the culture is raised to the air-medium interface, and it is fed from below. Such cultures have been

(Text continues on p. 256)

Figure 12 Early timepoint integrin staining on cytokine-injected volunteers. (A) α_2 staining 1 day after TNF-α injection; (B) β_1 staining 4 hours after IL-1 injection; (C) α_3 staining 24 hours after IFN-γ injection; and (D) α_3 staining 48 hours after IFN-γ injection.

Scale bar = 50μm.

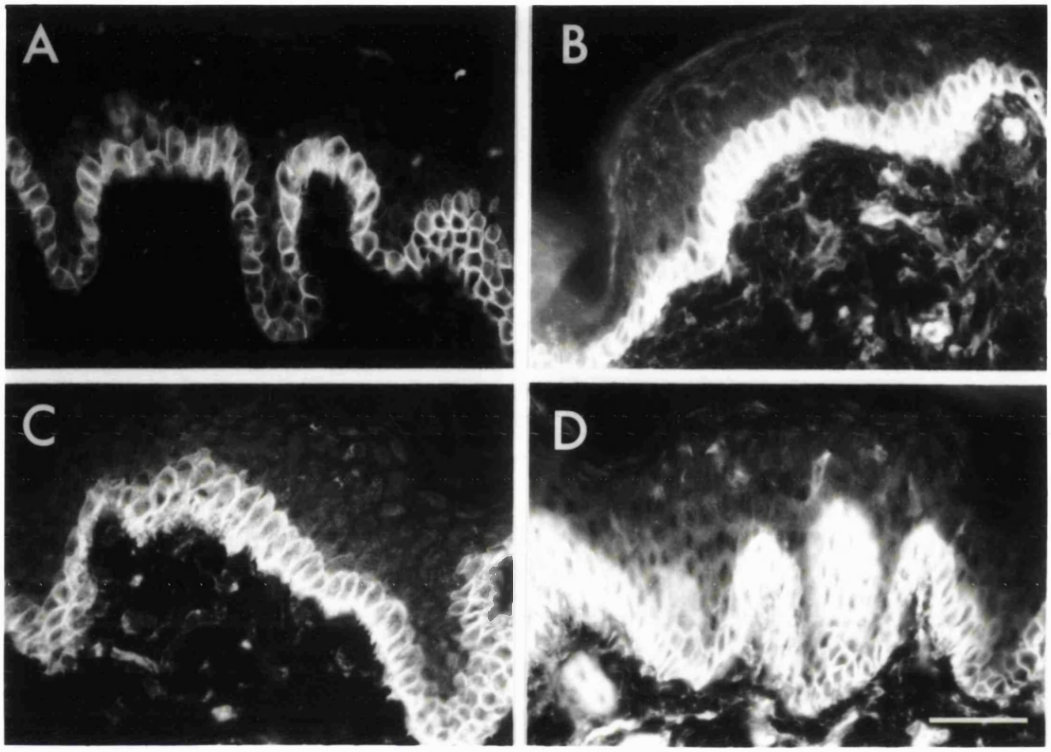

A
B
C
D

Figure 13 Late timepoint (5 days after injection) integrin staining on cytokine-injected volunteers. (A) α_2 staining after TNF-α injection; (B) α_2 on same volunteer after PBS injection; (C) α_6 staining after IFN-γ injection; and (D) β_1 staining after IFN-γ staining.

Scale bar = 50μm.

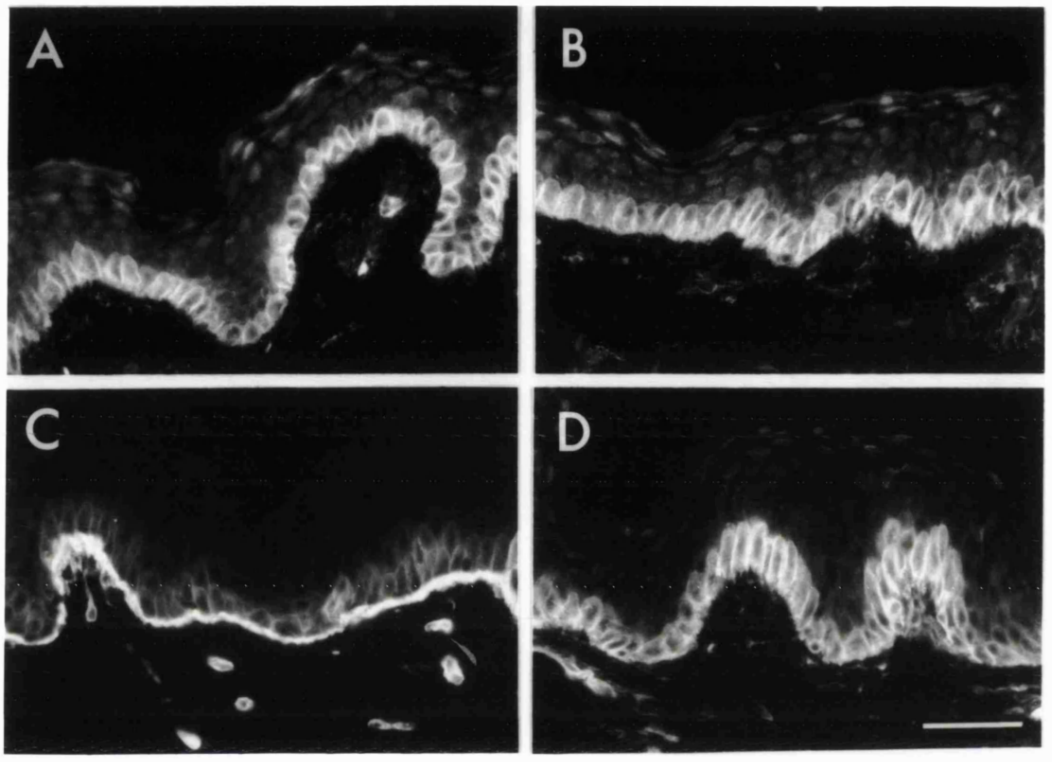
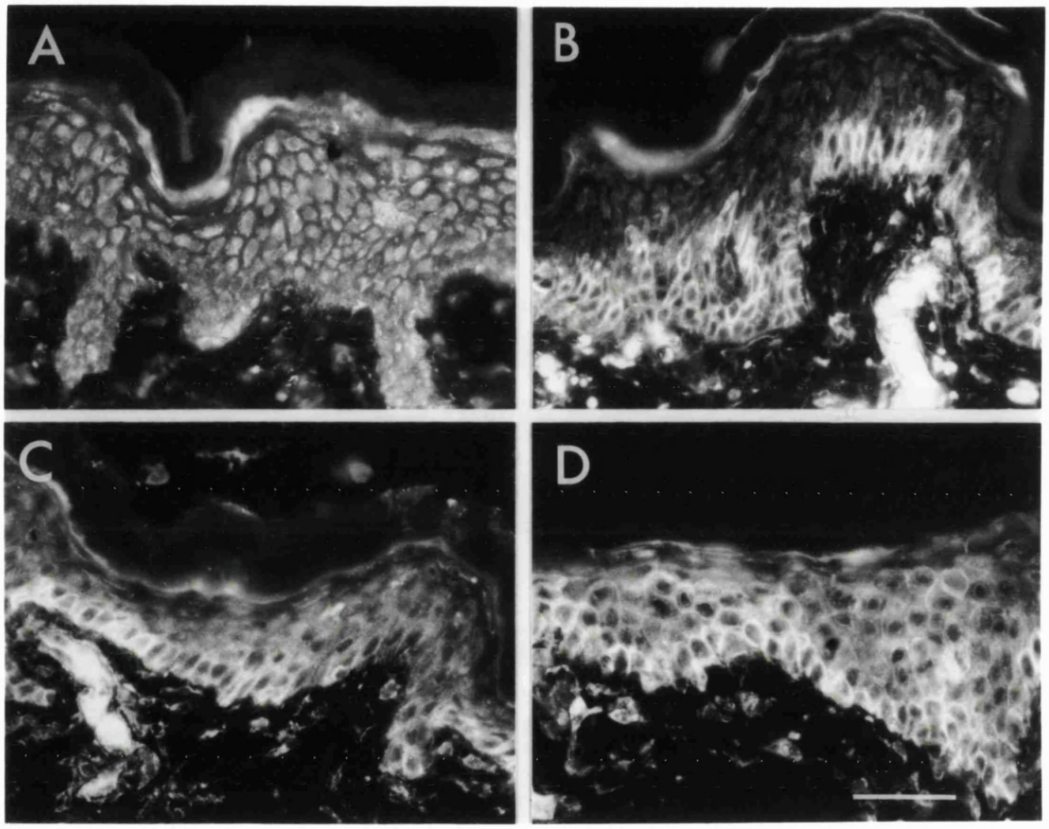

A
B
C
D

Figure 14 ICAM-staining in cytokine-injected volunteers. (A) control PBS injected; (B) approximately 24 hours after TNF-α injection; (C) 24 hours after IFN-γ injection; and (D) 48 hours after IFN-γ injection. Note intracellular staining in both cytokine-injected and control volunteers.

Scale bar = 50μm.


A
B
C
D

demonstrated to reproduce many of the features of epidermis *in vivo* (Bell *et al*, 1981; Asselineau *et al*, 1986; Kopan *et al*, 1987; Asselineau *et al*, 1989; Choi and Fuchs 1990) and better histological differentiation than in R & G cultures.

1 week old (raised to the air interface at 4 days and then cultured for 1 week), untreated raft cultures showed suprabasal staining. Keeping the raft culture submerged did not eliminate the suprabasal staining. Retinoic acid has been reported to induce $\alpha_5\beta_1$ expression in suprabasal keratinocyte in rafts (Asselineau *et al*, 1989). Using delipidized serum,which is depleted of retinoic acid, I was unable to suppress suprabasal staining at one week after raising (not shown). I tested IFN-γ, TGF-β, and IL-1β in air-exposed rafts and obtained the same results: the cultures expressed integrins suprabasally.

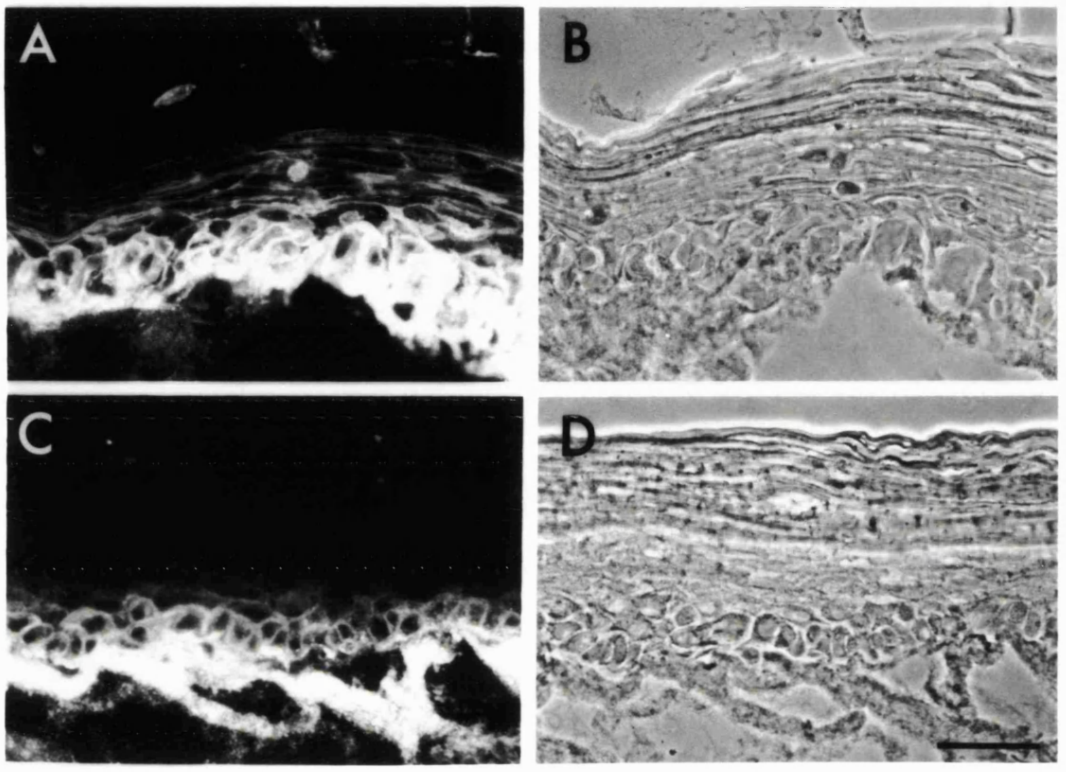
I varied the raft culture conditions in a number of ways (see Table 1) and found that suprabasal expression was transient: under all conditions, integrin expression was suprabasal at one week after raising (Fig. 15A), and predominantly basal two weeks after (Fig. 15C). No differences between standard keratinocyte feeder cells (mitotically-inactivated J2) or mitotically active dermal fibroblasts (HDF), in three different collagen-based substrates (more fully described in Chapter 2). Furthermore, I observed the same transient integrin expression pattern in cultures depleted of vitamin A, either air-exposed or submerged.

Table 1. Integrin expression pattern in raft cultures under different culture conditions.

	Suprabasal integrin at:	
Culture type	1 week	2 weeks
J2 feeders/Vitrogen, raised	+	-
J2 feeders/Vitrogen, delipidized medium, raised	+	-
J2 feeders/Vitrogen, submerged	+	-
J2 feeders/Vitrogen, delipidized medium, submerged	+	-
J2 feeders/Cellagen, raised	+	-
HDF feeders/RTC, raised	+	-
J2 feeders/Vitrogen, raised + IFN-γ, TGF-β, or IL-1β	+	-

Figure 15 β_1 integrin expression in keratinocyte raft cultures. Immunofluorescence labeling of β_1 integrin at (A) one week after raising to the air/medium interface; and (C) two weeks after raising to the air/medium interface. (B) and (D) are corresponding phase contrast micrographs illustrating the morphology. Remnants of collagen are evident below the basal keratinocytes.

Scale bar = 50μm.


A
B
C
D

Since suprabasal integrin expression in the rafts does not appear to be due to cytokines, but rather is an intrinsic property of the rafts, further experiments are in progress to address this. There may be a proliferative difference between 1 and 2 weeks after raising to the air/medium interface; I have labeled rafts with BrdU, a DNA nucleotide analog, to quantitate proliferation at 1 and 2 weeks. *In situ* hybridization is also in progress, to determine if suprabasal keratinocytes at 1 week are synthesizing integrin mRNA. Other adhesion molecules and markers of differentiation may show differential expression at 1 and 2 weeks. E-cadherin localization does not appear to be altered in the rafts (not shown), but P-cadherin, for example, may be expressed on suprabasal cells, analogous to the integrins. Involucrin expression is altered in hyperproliferative conditions (Chapter 4) and may exhibit the same pattern in the rafts at 1 week.

5.3 Discussion

I have tested the effects of several cytokines on the expression of keratinocyte integrins both *in vitro* and *in vivo*. Generally minor effects on basal keratinocyte integrin expression were detected; suprabasal integrin expression was not induced either *in vitro* or *in vivo* by the cytokines tested. Keratinocyte raft cultures, however, did transiently express integrins suprabasally. These results suggest that the suprabasal integrin expression I have detected both in wound healing and in psoriasis (Chapter 3) is not induced by cytokines, and does not require an inflammatory reaction.

5.3.1 Flow cytometric analysis of integrin expression

Flow cytometry was used to give an indication of the surface integrin expression intensity and whether integrins were expressed on suprabasal keratinocytes or not. Analysis of integrin expression by flow cytometry indicated that both increases and decreases were possible (Figs. 1-4). However, integrin expression changes on cytokine treatment were not consistent. This suggests that there is some uncontrolled factor which is influencing the level of integrin expression. One complicating factor, culture confluence, was controlled for as much as possible by using subconfluent cultures consistently. The same keratinocyte strain was used for the majority of experiments; pooling of results obtained for a single strain (not shown) likewise indicated considerable fluctuation in integrin expression on cytokine treatment. The time of treatment or level of other cytokines (those in added serum and supplements) did not have a significant effect on integrin expression. The induction of differentiation varied somewhat between experiments, but differentiation was consistently induced by most of the cytokines. On balance, the results must be taken to indicate that there are only minor changes in integrin expression, which are apparently not biologically relevant, as judged by the adhesion assay results. However, the variation in integrin expression, considerable in some instances, implies that cytokines can induce significant changes in integrin expression, but a confounding factor must be controlled for. Given that keratinocyte cultures are a complex population of cells in varying stages of terminal differentiation, it will be

difficult to separate, for example, the proliferative and differentiative effects from effects of cytokines on integrins.

The results were calculated using the modal value as I wanted to analyze cytokine effects on basal keratinocytes, the major population, once it was apparent that suprabasal expression was not induced. However, this analysis method is subject to error if the peak is not smooth, i.e. a "spike" which is not in the center of the peak can alter the result. We are now in the process of re-analyzing the data on the basis of the mean fluorescence intensity of the central portion of the major peak. This may yield more consistent results, and should not be subject to slight aberrations in the peak shape.

A *caveat* which must be considered when judging quantitative changes of integrin surface expression is the limitations of the antibodies used to detect the integrins. Changes in conformation, which may or may not be functionally significant, can increase or decrease the amount of antibody binding even in the absence of any quantitative changes in integrin level. In other words, flow cytometry is subject to qualitative as well as quantitive changes in integrin expression.

5.3.2 Integrin protein expression and function

Immunoprecipitation analysis indicated that there were no large changes in β_1 integrin synthesis or maturation rate after cytokine treatment (Figs. 5 and 6). It is possible that small changes were occurring; quantitation of the bands would be necessary to resolve this. Since the antibody used for immunoprecipitation recognizes the extracellular domain of β_1, it is unlikely that increased expression of alternatively spliced β_1 (Altruda *et al*, 1990; Languino and Ruoslahti 1992) would not be detected.

As discussed above, functional modifications of integrins are possible in the absence of changes in cell surface expression. I therefore examined integrin function as judged by adhesion to specific substrates. Adhesion of IFN-γ or TGF-β treated keratinocytes to 10μg ECM was approximately the same as control cells, or slightly increased. The small

increases in adhesion are consistent with the small increases or no change observed in integrin protein measured by immunoprecipitation and flow cytometry. However, it is important to stress that the differentiative effects of the cytokines used could distort the adhesion results somewhat. Since the adhesion assays are expressed as a percentage of the cells added that adhere, and since only basal cells are capable of adhering, agents that decrease the proportion of basal cells will in and of themselves reduce the percentage of adherent cells. Since both IFN-γ and TGF-β induce differentiation, measured by the percentage of involucrin positive keratinocytes, the actual basal cell adhesion after cytokine treatment is likely to be somewhat higher than that measured. However, even adding the maximum induction of differentiation of about 20% still results in a maximum increase of adhesion in the range of 50%.

5.3.3 Integrin mRNA levels

Northern blot analysis of integrin mRNA expression on cytokine treatment indicated that both IFN-γ and IL-1β were not capable of inducing major changes in steady-state β_1 message compared to control cells (Fig. 7). This is consistent with a lack of changes in integrin protein synthesis, although present evidence indicates that integrin regulation can occur at both the transcriptional and post-translational levels (Hotchin and Watt 1992). Dose-dependent ICAM-1 mRNA expression in IFN-γ treated keratinocytes indicated that the amount of IFN-γ used was sufficient to induce a response from the keratinocytes (Fig. 8).

5.3.4 Suprabasal keratinocyte integrin induction *in vitro* and *in vivo*

I assessed the keratinocyte integrin localization in two types of keratinocyte cultures treated with cytokines and in volunteers injected with cytokines. Submerged keratinocyte cultures on plastic, in which integrin expression is restricted to the basal layer — as it is in normal epidermis — still restricted integrin expression to the basal layer after cytokine treatment. Keratinocyte raft cultures expressed integrins suprabasally in the absence of cytokines and are discussed separately below.

The integrin expression pattern was also examined *in vivo*, in volunteers injected with a single cytokine (Camp *et al*, 1990; Barker *et al*, 1992). Although it is impossible to attribute the results to a single cytokine, as other cytokines will have been induced both by the injected cytokine and the inflammatory infiltrate, neither IFN-γ, TNF-α, nor IL-1α was able to induce suprabasal integrin expression. The IL-1α biopsy time of 4 hours after injection may not have been enough time to observe effects on integrins at the cell surface, but the biopsy times for IFN-γ and TNF-α, 24 hours to 5 days, should have been ample time to allow suprabasal expression (suprabasal integrin expression during wound healing occurs within 4 days; Chapter 4). ICAM-1 expression in both IFN-γ and TNF-α-injected volunteers indicated that the amount injected was sufficient to induce an epidermal response.

Keratinocyte raft cultures, which are the best *in vitro* equivalent of the skin (Fuchs 1990), expressed integrins suprabasally one week after raising to the air interface, in the absence of any specific cytokine treatment beyond that in normal keratinocyte medium. However, by two weeks after raising, integrin expression was generally confined to the basal layer, though there was still some suprabasal staining. Different feeder cells and collagen sources did not eliminate the suprabasal staining, nor did elimination of retinoic acid. It therefore appears that the transient suprabasal integrin expression is an intrinsic feature of the raft cultures. The factors released by the feeder cells when in a collagen matrix may be different than when they are attached to plastic, and may resemble factors present in wound healing and psoriasis. The degree of proliferation at 1 and 2 weeks may provide some clues, and I am in the process of determining this by BrdU incorporation. Involucrin expression is altered in hyperproliferative conditions, and it will be interesting to examine its localization in the rafts. Although I was unable to resolve the question of suprabasal integrin gene transcription during wound healing (Chapter 4), I am examining the *in situ* integrin expression pattern in the rafts.

There is one other example to date in which markers of terminal differentiation and integrins can be co-expressed in keratinocytes. The raft cultures are morphologically more similar to skin, but it has been demonstrated that plastic-attached keratinocytes, kept as a monolayer by culturing in low calcium levels are able to express integrins and

involucrin, a terminal differentiation marker, simultaneously (Kairbaan Hodivala, Keratinocyte Lab, ICRF). This situation is, in terms of the marker co-localization, identical to wound healing and psoriasis. If calcium is added back to the cultures, re-stratification occurs and integrin and involucrin expression once more become mutually exclusive. However, if a combination of P- and E-cadherin antibodies is added when calcium is added, integrins and involucrin continue to be co-expressed for much longer. This apparent communication between adhesion molecules may provide some clues to the causes of suprabasal integrin expression, which can be investigated with the raft cultures.

In conclusion, several lines of evidence point against a role for cytokines in inducing suprabasal integrin expression in the epidermis during wound healing and in psoriasis. The array of inflammatory cytokines present in both wound healing and psoriasis, and their localization and presentation, are more complex than what can be reproduced *in vitro*. However, *in vivo*, injection of IFN-γ, TNF-α, or IL-1α (each of which will have induced additional cytokines) was unable to induce suprabasal expression.

It seems likely therefore that a condition other than inflammatory cytokine expression, that wound healing, psoriasis, and 1-week rafts have in common with each other but not with normal skin or plastic-attached keratinocyte cultures, is responsible for suprabasal integrin expression. The conditions for suprabasal integrin expression are apparently reproduced in keratinocyte raft cultures, and these will provide a provide a model for further study of this intriguing phenomenon.

Appendix 2

Preliminary results: Strategy for the identification and isolation of a keratinocyte master control gene

Introduction

One of the earliest events in the development of an organism is commitment to particular pathways of differentiation, driving pluripotential cells to become specialized tissues (Alberts *et al*, 1989). Although some pathways of differentiation are due at least in part to "default", i.e. absence of expression of a particular control gene, it is likely that at the branching off of a differentiated cell type, a gene able in itself to turn on the entire cascade of differentiative steps should exist and be expressed at the earliest divergence. Such a gene, *myoD*[1], although now doubted to be a true master control gene, is able to turn on the cascade of gene expression necessary for differentiation into myoblasts and ultimately myotubes.

The myoblast determination gene, MyoD

MyoD was originally identified as a gene which, when transfected into the $10T^1/_2$ cell line, was sufficient on its own to convert the cells into muscle cells which not only expressed the typical markers of muscle cells bet were also able to fuse into myotubes (reviewed in (Weintraub *et al*, 1991)). Using *myoD* as a paradigm, it seems likely that such a gene should exist for keratinocytes (Fuchs 1990), which alone could commit a competent epidermal precursor to become a keratinocyte. However, it is possible that the epidermal pathway is determined by default — this has been demonstrated for *Xenopus*. The information to turn on the expression of the regulatory genes may come from spatial organization (e.g. homeobox gene expression (De Robertis *et al*, 1990)), or factors such as morphogenic gradients (e.g. retinoic acid (Tabin 1991)), or most likely a combination of factors.

The *myoD* gene was first identified in 1987, by its ability to drive $10T^1/_2$ fibroblasts cells down the myoblast lineage, when *myoD* was under the control of a strong promoter/enhancer (Lassar *et al*, 1986; Davis *et al*, 1987). Subsequently, it has been found to be able to drive a variety of cell

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[1] *myoD* = gene, MyoD = protein

types down the myoblast lineage, although it is only naturally expressed in skeletal muscle, being repressed in other cell types — including cardiac and smooth muscle. MyoD appears to act by binding control regions of muscle-specific genes, using a helix-loop-helix domain; it must bind upstream in at least two sites to activate transcription (Murre *et al*, 1989). MyoD must heterodimerize with another helix-loop-helix protein, encoded by the E2A gene, to bind its recognition site efficiently and activate muscle-specific genes. There are other members of this family — e.g. *myf-5* (Braun *et al*, 1989) and myogenin (Wright *et al*, 1989)— but it is not clear if these genes have different biological functions from *myoD*. Each gene on its own is able to turn on the others, including *myoD*, and can initiate myogenic commitment; they appear to reinforce each other, making commitment irreversible.

The activity of MyoD can be repressed: in myoblasts, expression of *myoD* in the absence of serum results in MyoD binding its recognition site and subsequent formation of myotubes (reviewed in (Weintraub *et al*, 1991). However, in the presence of serum, *myoD* expression does not result in differentiation. The product of the *Id* (inhibitor of differentiation) gene, expressed in most cells, can bind MyoD and render it unable to bind its enhancer sequence, but *Id* expression declines substantially in the absence of serum. Thus, there are several levels of control beyond the presence of a recognition site, which cannot be the only regulatory factor.

The upstream enhancer consensus site for MyoD, CA--TG, is also present in the enhancers of other cell types, where it can be bound by other transcriptional activators, e.g. *achaete-scute*, a neurogenic master regulator in *Drosophila*. It is therefore still unclear how specificity is conferred, i.e. why *myoD* expression doesn't also turn on neural-specific proteins. There is evidence that a domain spanning only a few amino acids in the basic region of myoD, when mutated, still allows binding to the consensus sequence, but prevents transcriptional activation; thus, the muscle-specific DNA binding region of *myoD* may be regulated by the basic region.

Embryo stem cells

The interest in and isolation of embryo stem (ES) cells derived from work on teratocarcinoma cells (also known as embryonal carcinoma, or EC, cells) which demonstrated the multipotential nature of EC cells (Martin 1980). Teratocarcinomas are tumors formed by germ cells; they can also be induced simply by injecting germ cells into nude mice. Teratocarcinoma cells are capable of differentiating into multiple cell types, but they are rarely able to participate in germ cell formation if injected into a mouse blastula, although they can form parts of most other organs.

Embryo stem (ES) cells are derived from the inner cell mass of the mouse embryo, i.e. those cells which will go on to form the mouse (Gossler *et al*, 1986; Robertson *et al*, 1986). When correctly maintained in culture, ES cells can be injected into a mouse blastocyst and will take part in embryogenesis, forming a chimeric mouse. Importantly, the injected ES cells can also integrate into the germ cell lineage, enabling changes in the ES cells (experimentally induced by e.g. homologous recombination with transfected foreign DNA) to be propagated in the chimera's offspring. This allows a high frequency of heterozygotes, as only ES cells containing the mutation/change are injected, since they can be selected for *in vitro*.

The use of ES cells has so far yielded a great deal of information, most significantly by the discovery that a gene of interest could be "knocked out" in the ES cells *in vitro* by the transfection of a slightly mutated form of the gene, which can homologously recombine — at low frequency — with the endogenous gene, resulting in a cell carrying the altered allele (Thomas and Capecchi 1987; Doetschmann *et al*, 1988; Schwartzberg *et al*, 1989). When injected into a mouse embryo, the mutant cells can form part of the embryo and result in a chimeric mouse, regions of which, including the cells of the germ line, will carry the mutant gene. Breeding chimeric mice results in a homozygote and allows analysis of developmental effects of the mutation.

Embryo stem cells are therefore totipotent, i.e. they can become any differentiated cell type in the mouse. This implies that ES cells are unrestricted and are competent to become any cell type. I needed a cell

line competent to become an epithelial cell, i.e. an ectodermal derivative. Embryo stem cells are relatively easy to manipulate as a target cell for transfection, and additionally offer the advantages outlined above for future work, to exploit any interesting genes found by transfection.

Approach

I took the approach of Pinney *et al* (Pinney *et al*, 1988), with modifications, to identify and isolate a putative keratinocyte determination gene. I set out to transfect mouse embryo stem cells with a human genomic DNA library. By selecting for the appropriate mouse-derived keratinocyte-specific marker, I hoped to isolate clones which contained a sequence which itself did not encode for particular keratinocyte markers, but was able to induce expression of these markers, and in essence, force the ES cells down the keratinocyte pathway. Embryo stem cells are capable of becoming keratinocytes, as demonstrated by implantation of ES cells into nude mice, which form carcinomas containing epidermal cysts (R. Beddington, pers. comm.; (Martin 1980)).

Results

I first learned how to culture ES cells without spontaneous differentiation. ES cells differentiate if allowed to grow to too high a density, if the feeders are poor, or if the ES cells are not completely disaggregated at passaging (culture method described in Chapter 5 and (Robertson 1987)). Initially, the CC3 line was used, but this line grew poorly, and I obtained another strain, CCE, which grew much better. After early difficulties, mostly due to not disaggregating the cells vigorously enough at passaging, the cells grew well, requiring passage every three days, and continued to exhibit typically undifferentiated morphology (not shown, but illustrated in (Robertson 1987)), ultimately up to passage 45, the highest attempted.

A source of genomic DNA to use for transfection was obtained in the form of a cosmid human genomic library. A cosmid library was chosen, as it offered advantages over a lambda phage (λ) genomic library, in that: cosmids are able to carry up to 45 Kb of DNA, as opposed to ~8Kb in λ, and therefore fewer clones would have to be screened to find positive ones; and in that the cosmid used (pEMBL8) was a shuttle vector, which could be expressed both in eukaryotic and prokaryotic cells, offering greater flexibility during subsequent manipulation. The cosmid DNA was isolated by growing up the isolates (four in total, representing each gene in the human genome at least once) on large agarose-Luria broth plates, at a density low enough to ensure that each colony could grow to a reasonable size; the DNA was extracted using standard phenol extraction, taking care not to shear the DNA. The size of the cosmid isolated DNA was confirmed to be in the range of 40-45Kb by gel electrophoresis, indicating that a significant amount of shearing during isolation had not occurred.

To prepare for the transfection of the cosmid DNA, several methods were investigated, but ultimately electroporation was chosen, because it was rapid and efficient. Because the ES cells would be selected for presence of cosmid sequences by selection in G418 (also called neo; Gibco-BRL), as $G418^r$ was encoded on the cosmid, the ES feeders (STO cells) would also have to be resistant to G418.

Inherent resistance of STO to G418

It was first necessary to determine the level of G418 which would kill untransfected STO cells, in order to use a sufficiently high concentration to select resistant clones once transfected. Two types of selection were used: ability of clonal density STO to survive, and ability of near-confluent STO to survive, in varying concentrations of G418. G418 concentrations of 200, 400, 600, and 800μg/ml were used, plus a control that was untreated. Passage 5 STO cells were used, in 35-mm gelatin-coated dishes.

Confluent survival

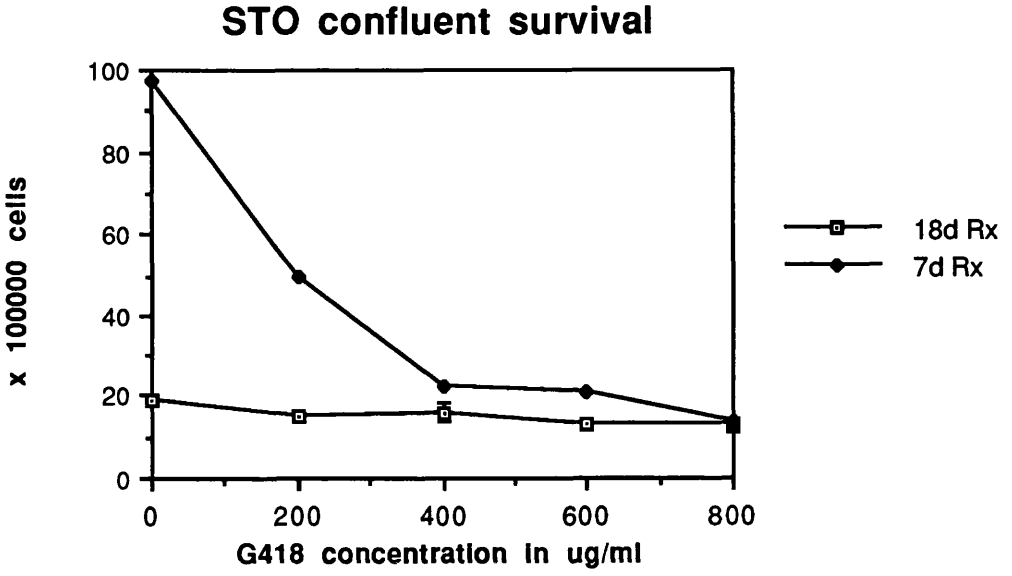
3.9×10^5 cells were plated in 35mm tissue culture dishes and allowed to grow for one day. Triplicate wells were counted for the starting density, and duplicate wells were treated, at concentrations indicated above. The cell number had virtually doubled in 24 hours. Medium was changed, with G418 added as appropriate, after 3 days, and every 2 days thereafter. By 7 days, STO at all G418 concentrations, but not controls, looked somewhat rounded and vacuolated; by 18 days, higher concentrations of G418 resulted in cultures that had areas bare of cells, with floating cells, and generally poor-looking cells. Cultures were trypsinized and counted at 7 and 18 days. The results are plotted as a survival curve in Fig 1A. Cells appeared able to resist, to some degree, the effects of G418 at all concentrations until at least day 7; after this cell numbers declined in all cultures. A concentration of 500μg/ml was chosen for selection as this concentration would be predicted to be sufficient to kill all untransfected cells.

Clonal survival

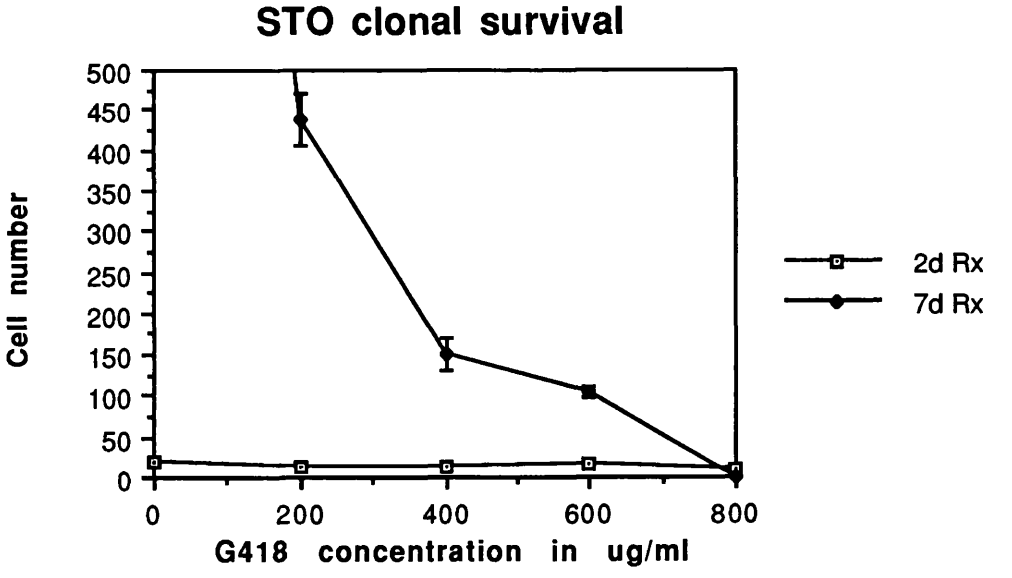
The ability of low cell numbers to survive in G418, to reduce the confounding effects of cell cross-feeding, was also tested. Triplicate wells of 50, 100, and 150 cells were plated and treatment with G418, as above, was started immediately. Two days later, the 150-cell wells were roughly counted; the other wells had too few cells for accurate counting. A single

Figure 1 Survival of STO cells at (A) confluent and (B) clonal densities in different concentrations of G418. "Rx" is treatment.

A.


STO confluent survival
x 100000 cells
100
80
60
40
20
0
0
200
400
600
800
G418 concentration in ug/ml
18d Rx
7d Rx

B.


STO clonal survival
Cell number
500
450
400
350
300
250
200
150
100
50
0
0
200
400
600
800
G418 concentration in ug/ml
2d Rx
7d Rx

visual count of triplicate wells, along the diameter of the plate, is listed in Table 2. Cells at 800μg/ml looked rounded and vacuolated. Four days after seeding, medium was changed, with fresh G418. Seven days after seeding, the plates were fixed in methanol and stained with crystal violet for cell counting. The 50 and 100-cell wells had too few cells for accurate counting; only the 150-cell triplicate wells were counted for all cells in the well. The results are plotted in Fig 1B. 400μg/ml was sufficient to prevent further cell division; 200μg/ml was sufficient to slow growth.

Inherent resistance of CCE to G418

As discussed for STO cells, above, the level of G418 sufficient to kill untransfected ES cells needed to be determined. The method used was as above, with CCE passage 3 cultured on X-irradiated STO feeders, except as noted below.

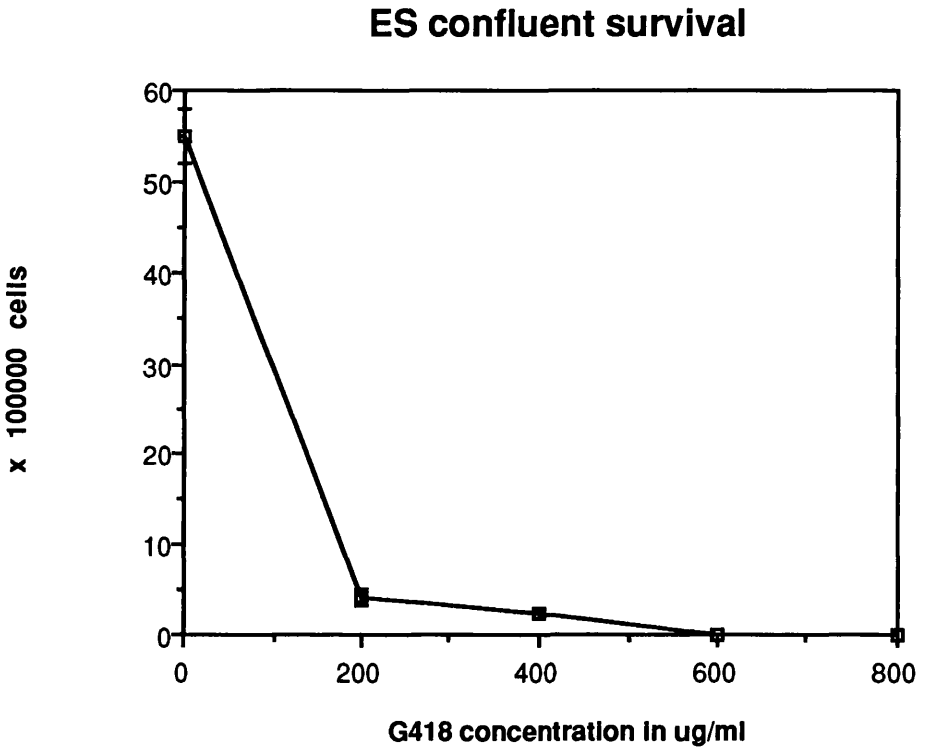
Confluent survival

ES cells were plated on 35mm dishes, allowed to grow for one day without selection, then medium containing the appropriate concentration of G418 was added. G418 concentrations of 200-800 μg/ml were added and cells were cultured for 6 days with one medium change. After three days, untreated cultures were confluent, 200μg/ml had some few floating cells, and with increasing G418 concentrations, greater numbers of dead cells were evident. After six days, untreated cultures were extremely dense; from 200μg/ml and up cultures appeared unhealthy. Cultures were trypsinized and counted, both using trypan blue exclusion. The results, illustrated in Fig. 2, suggested that concentrations of G418 as low as 200μg/ml were sufficient to kill most or all ES cells using these conditions. A concentration of 200μg/ml G418 was chosen for future selection.

Clonal survival

1.5×10^6 cells were plated on 35mm dishes, allowed to grow for one day without selection, then medium containing the appropriate concentration

Figure 2 Survival of ES cells at confluent density in different concentrations of G418. Total number of trypan-blue unstained cells remaining after 6 days.


ES confluent survival
x 100000 cells
60
50
40
30
20
10
0
0
200
400
600
800
G418 concentration in ug/ml

of G418 was added. Medium was changed daily, using fresh G418. After three days, cells were passaged onto new feeders at 100, 200, and 300 cells per well and selection was maintained. After six days, few cells were evident; after 11 days, some colonies were evident in dishes containing 300 untreated cells. There were no cells at any concentration of G418. Apparently, using this approach, isolated cells cannot survive in a G418 concentration as low as 200μg/ml.

Preparation of G418-resistant STO cells

G418 interferes with the function of 80S ribosomes and therefore blocks protein synthesis (Sambrook *et al*, 1989). Several vectors, carrying the Tn5 phosphotransferase gene, coding for a protein allowing growth in G418, are available. pSV2neo, carrying Tn5 driven by the SV40 promoter/enhancer (Southern and Berg 1982), was then obtained. Rather than develop an electroporation protocol for the STO cells, lipofection was used to generate resistant feeders. This technique uses liposome-mediated transfer of DNA molecules to introduce the transfected sequences into the cell. DNA concentrations of 0, 1, 5, and 10μg of *Eco* RI-linearized, purified pSV2neo DNA + 40μg Lipofectin were used, as detailed in Chapter 5. Cells were allowed to grow to confluence (5 days), and selection in 500μg/ml G418 was started.

To generate a homogeneous population, cloning by single cells and low cell numbers (150 or 300 cells) was attempted. However, all cells died, at all selection conditions, so the polyclonal population was used, as all cells were resistant to G418. For future experiments, the STO transfected with 5μg of pSV2neo were used; they were designated STOneo5. Genomic DNA was extracted from all cells (normal STO, lipofectin-only treated STO, and 1, 5, 10μg transfected) for analysis by Southern blotting to confirm integrated *neo* (not shown).

ES electroporation

The method of (Schwartzberg *et al*, 1989) was used, as detailed in Chapter 5. Following electroporation, attached cells usually appeared

healthy. After several electroporation runs, resistant colonies were consistently obtained; efficiency of transfection was calculated to be between between 3.4×10^{-5} and 1×10^{-4}. Resistant cells were passaged from then on, in the presence of G418, and always using G418-resistant STO as feeders.

After electroporation with pSV2neo was established, two different fractions of the cosmid genomic library (25μg of fractions A & E, linearized with *Mlu* I) were electroporated and cells were selected in G418 as above. Although the cosmids are fairly large (~45kb), electroporation has been demonstrated to be effective with cosmids (Andreason and Evans 1988). Transfectants were designated CCE-A and CCE-E and placed under selection. Two weeks later, transfected cells were seeded onto coverslips, in FAD + HICE + 10% FCS, as a preliminary survey for epithelial-specific gene expression, i.e. keratin 14 and integrin α_6. Originally, the only integrin antibody we had that would recognize mouse cells was the anti-α_6 GoH3. Subsequently, I obtained a mouse-specific anti-β_4 (Kennel *et al*, 1986), which should identify epithelia only, as β_4 has not been demonstrated in other tissues.

Southern analysis for integrated *neo*

To check if pSV2neo or the cosmids had integrated in the genome, Southern analysis was performed. Although transient transfection would result in G418 resistance, non-integrated plasmids/cosmids would not be isolated by the genomic DNA isolation technique. DNA extracted from untransfected CCE, pSV2neo-transfected CCE, CCE-A and CCE-E was analyzed. In all cases, however, neither pSV2neo-nor pcos8-integrated DNA could be detected (not shown).

DNA topology is an important factor in successful integration. In some cases, supercoiled DNA may be more efficiently taken up and integrated (Andreason and Evans 1988). Therefore, the efficiency of transfection using 25μg of linearized pcos8, linearized pSV2neo, or uncut (supercoiled) pcos8 was compared. Cell survival was comparable, with ~50% plating efficiency. Selection was performed in 300μg/ml G418 (as suggested in (Robertson 1990)), and cell numbers at each passage were

compared for transfected cells with or without G418. Initially, cell numbers in G418 dropped considerably, compared to no selection, but approximately two weeks following electroporation, cell numbers were approaching equivalence. At this point, the G418 concentration was increased to 484μg/ml. This higher concentration reduced the cell numbers again, compared to unselected cells. By three weeks following transfection, mock-and supercoiled pcos8-transfected cells in G418 were virtually all dead, while cells transfected with linear pcos8 were dying, and linear pSV2neo cells were still growing. At this point, representative flasks were harvested and DNA was extracted. As shown in Fig. 3, untransfected CCE were negative for *neo*, while CCE transfected with pSV2neo produced a band that hybridized at ~5 kb, indicating that CCE contained integrated *neo*. Although long-term propagation of unintegrated plasmid should be impossible, since it does not contain the eukaryotic sequences necessary for replication, if it were present it would run at 5.7kb (see control plasmid track) and not at 5kb (transfected CCE).

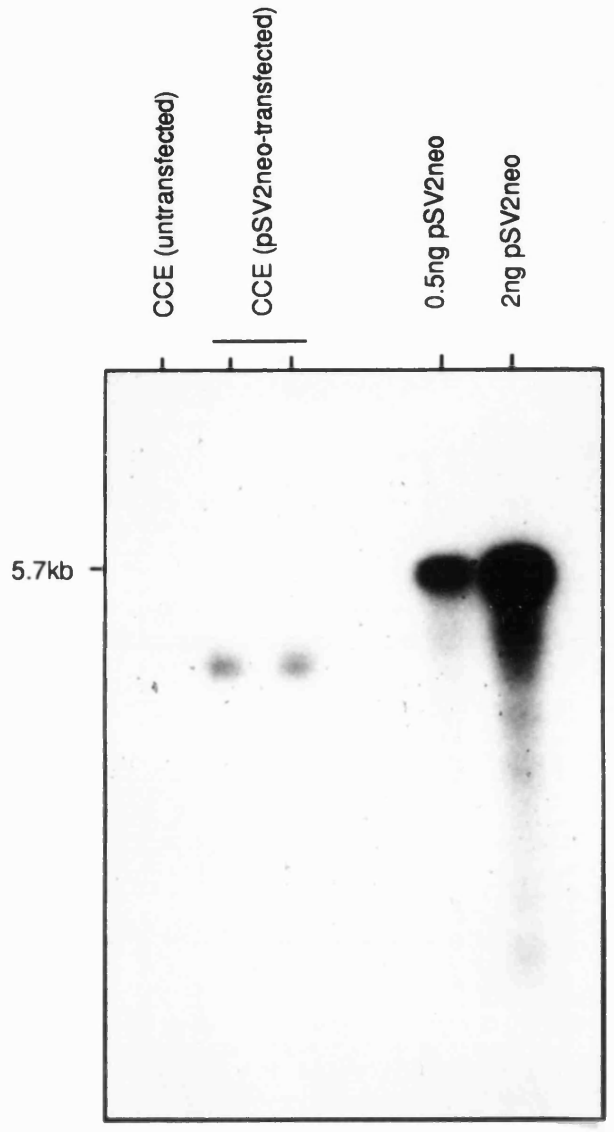
Repeated attempts at transfection with pcos8 were unsuccessful, as judged by G418 resistance.

Screen for epithelial markers

I had previously checked for both K14 (a marker for stratified epithelia) and K18 (a marker for unstratified) expression, at 3 and 10 days of culture, in untransfected CCE. At both timepoints, CCE were positive for K18, as reported previously (Jackson *et al*, 1980; Plancha *et al*, 1989), and were negative for K14 (not shown). Therefore, expression of K14 in transfected cells would suggest that either the K14 gene, or possibly a higher level inducer of K14 expression (endogenous, mouse K14) had been introduced. A species-specific antibody would be necessary to distinguish between these two possibilities; induction of endogenous K14 would be more encouraging.

Preliminary screening of CCE-A indicated that K14 was not expressed (not shown), but at this point I was not convinced that the cells were truly transfected, so further analysis of this clone of cosmid library transfected cells was not performed.

Figure 3 Southern analysis of 25μg of pSV2neo-transfected ES cell DNA, digested with *Hind* III. Untransfected CCE are compared to duplicate tracks of CCE transfected with linear pSV2neo. Restriction-digested pSV2neo is shown as a positive control. Samples were probed with the 0.8kb *Pvu* II fragment of pSV2neo, representing only *neo* sequences.


CCE (untransfected)
CCE (pSV2neo-transfected)
0.5ng pSV2neo
2ng pSV2neo
5.7kb

Discussion and Conclusions

I established the selection conditions for STO feeders and ES cells, and ultimately was successful in transfecting the ES cells to G418 resistance using pSV2neo. The source of the library I had chosen to transfect appeared to present some problems, however. I was unable to transfect ES cells successfully with the cosmid itself or cosmid containing human genomic DNA sequences. This could be due to technical problems, but there were other indicators that suggested another library would be a better choice. In discussions with the originator of pcos8, Dr. Alaister Craig, and others, it was suggested that the ES cells might be able to repress *neo* expression; this meant another cosmid vector would be better. Indeed, it has been documented that undifferentiated embryonal carcinoma (EC) cells can repress the expression of several promoters (Hasegawa *et al*, 1990). Although the expression of the transfected genomic sequences would, at least in part, be regulated by the endogenous promoter attached to the gene, selection would not be possible if *neo* expression were repressed. It is also possible that transfection was successful, but methylation inhibited *neo* expression and perhaps subsequent loss of the transfected DNA (Shinar *et al*, 1989).

It became clear that to ensure expression of the *neo* gene, another cosmid library was necessary. I attempted to obtain another human cosmid library (Lau and Kan 1984), with *neo* under a stronger promoter, that might have been better expressed in the ES cells, but unfortunately that library was not available. Since I was unable to obtain one, the only alternative was to generate my own. The time necessary to generate and characterize the library, coupled with the technical problems envisioned (e.g. selection time in G418 was sufficiently long that the ES cells would proliferate significantly and need passaging, therefore necessitating the screening of many more clones, some of which, although transfected, could grow slowly and be lost; and the unequivocal selection of potential keratinocytes) and the success of other projects, led us to decide that this project should be shelved. I think the approach is fundamentally workable. Given the tremendous value of a keratinocyte determination gene, this project is worth pursuing, although it is worth bearing in mind that there may not be a determination gene for keratinocytes. That no "master" regulatory genes other than MyoD have been found so far also

suggests that isolation of these genes is difficult, or that they may not actually be common.

Conclusions and prospects

Conclusions

There is reliable evidence that the integrins are important in regulating keratinocyte adhesion and terminal differentiation, and spatial organization of keratinocytes *in vitro.* I have examined the integrin expression patterns in two situations*in vivo* to see if there were changes in expression associated with the morphogenesis during development and during re-establishment of the epidermis in wound healing. I have found developmentally regulated expression of integrins during development, which appears to correlate with periods of epidermal morphogenesis, and established the usefulness of the fetal skin organ culture model for further investigation of the role of integrins during epidermal morphogenesis.

During epidermal wound healing, I found a dramatic change in the localization of integrins (i.e. suprabasal) in the epidermis, which is also found to a lesser degree in psoriasis and neoplastic conditions. One of the primary candidates for causing suprabasal expression, inflammatory cytokines, does not appear to induce any significant alterations in integrin expression or function in keratinocytes. However, the keratinocyte raft model does accurately reproduce the suprabasal expression, and provides a model for further examination of this potentially clinically important phenomenon.

Prospects

The fetal skin organ culture model will allow examination of the effects of RGD peptides and integrin-inhibitory antibodies, and will help clarify the role of integrins in establishing the epidermal architecture. Additionally, the observation that integrin expression in organ culture accurately reflects the pattern observed in vitro further strengthens the value of fetal skin organ culture for examining other developmentally regulated molecules.

The agent(s) responsible for suprabasal integrin expression may be identifiable using the keratinocyte raft model. *In situ* hybridization should resolve the question of the source of the suprabasal integrins (i.e. whether integrin genes are transcribed suprabasally). The issue of the role

of epidermal hyperproliferation in suprabasal integrin expression can also be addressed with the rafts. Further experiments in altering the dermal component of the rafts may also shed light on suprabasal integrin expression.

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