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Invited Review

Preservation of human skin structure and function in organ culture

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Summary. Human keratinocytes can be maintained in monolayer culture under serum-free conditions for an extended period of time. Under low Ca^{2+} conditions (e.g., 0.05-0.15 mM), an undifferentiated state is maintained and the cells proliferate optimally. When the Ca^{2+} concentration is raised to approximately 1.0 mM, differentiation occurs and growth slows. Human dermal fibroblasts can also be maintained in monolayer culture under serum-free conditions, but in contrast to keratinocytes, a physiological level of extracellular Ca^{2+} (above approximately 1.0 mM) is required. A variety of growth factors stimulate proliferation of both cell types but do not replace the Ca^{2+} requirement of the fibroblast population. All-trans retinoic acid also promotes proliferation of both cell types and, most interestingly, replaces the requirement for a physiological level of Ca^{2+} in the fibroblast cultures. Human skin can be maintained in organ culture for an extended period of time under serum-free conditions. Conditions optimized for fibroblast proliferation (either physiological Ca^{2+} or all-trans retinoic acid) are required. In the presence of culture conditions optimized for the epithelial cell component, both the epidermis and dermis rapidly lyse. These data suggest that the fibroblast is the critical component in maintaining homeostasis of skin, and that maintenance of the epidermis as well as the dermis depends on the viability and functioning of these cells.

Key words: Keratinocyte, Fibroblast, Skin organ culture, Epidermis, Dermis, Growth factors, Ca^{2+}, All-trans retinoic acid

Introduction

The skin forms the continuous external surface of the body. It consists of two major compartments; the upper layer, or epidermis, and the lower layer, or dermis. The epidermal layer is composed largely of squamous epithelial cells (keratinocytes). At the base of the epidermis are the proliferating keratinocytes. Above are the differentiating epithelial cells, organized into more or less well-defined morphological layers. Melanocytes are also found in the basal layer of the epidermis. The dermal layer is a thick, dense fibro-elastic connective tissue in which interstitial fibroblasts are embedded. A rich vascular network permeates the dermis (Harrist and Clark, 1994). The epidermis and dermis are separated by a thin, sheet-like extracellular matrix (ECM) structure known as the basement membrane. Major components of the basement membrane are laminin, type IV collagen and heparin sulfate proteoglycan (Fine, 1987). The major cellular elements of both compartments are readily visualized after hematoxylin and eosin staining (Fig. 1a). Connective tissue elements can also be visualized in hematoxylin and eosin-stained preparations (Fig. 1a), though special stains are useful for enhancing the visualization of the extracellular matrix. Transmission electron microscopy (Fig. 1b) is required to identify structural detail in the basement membrane and dermal connective tissue elements.

The skin has many functions. Protection of the organism against physical, chemical and biological stresses is its major function. The skin also serves as a water vapor barrier, has metabolic and immunologic functions and contains the receptors for sensation (Harrist and Clark, 1994).

The skin has proven to be a useful tissue with which to probe biological questions that are germane not only to the skin, itself, but to other tissues and organ systems. For example, the skin has been used to study the cellular and molecular basis of normal vs abnormal wound repair; to study mechanisms of inflammatory and immunological tissue injury; to elucidate the basis for benign and malignant hyper-proliferative diseases; and to study the natural ageing process as well as how exposure to environmental stresses such as ultraviolet light alters this process. Because the skin is amenable to topical treatments, it has also been used to assess potentially toxic and/or therapeutic properties of a number of agents which could not be evaluated systematically. While the skin appears to be a suitable model for all of these studies, it is our belief that understanding how normal homeostasis is maintained is

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a critical pre-requisite for understanding any of the physiological or patho-physiological changes that can occur. For this reason, a considerable effort has been spent to understand the cellular and molecular events which contribute to maintenance of structure and function of normal skin. *In vitro* studies with cells in monolayer culture and, in particular, with whole skin in organ culture have been useful for this. It is clear from such studies that cells of the dermis, especially dermal fibroblasts, are a critical element in preserving both the epidermis and dermis.

Maintenance of homeostasis in skin

Keratinocyte and fibroblast growth in monolayer culture: Role of growth factors and extracellular Ca^{2+}

The major cellular elements of skin - e.g., keratinocytes and fibroblasts - can be routinely grown in cell culture under serum-free conditions. Growth of neonatal and adult keratinocytes is stimulated by a number of different factors, including epidermal growth factor (EGF) and other growth factors that function through the EGF receptor (e.g., transforming growth factor-α, amphiregulin, heparin-binding EGF) (O'Keefe and Chiu, 1988; Elder et al., 1989; Ferriola et al., 1991; Cook et al., 1991a,b), by factors elaborated in the dermis, including insulin-like growth factor-1 (IGF-1) (Froesch et al., 1985; Krane et al., 1991; Neely et al., 1991), keratinocyte growth factor (KGF) (also known as fibroblast growth factor-7) (O'Keefe et al., 1988; Ristow and Messmer, 1988; Barreca et al., 1992) and hepatocyte growth factor (HGF) (Sato et al., 1995).

Keratinocytes from neonatal foreskin proliferate in the absence of exogenous growth factors, but cells from adult skin have a limited growth potential under basal conditions. Of interest, antibodies to the EGF receptor are potent inhibitors of keratinocyte growth in monolayer culture. They inhibit basal proliferation as well as EGF-stimulated growth, suggesting that keratinocyte growth occurring in the absence of exogenous growth factors is mediated through autocrine loops involving the EGF receptor (Varani et al., 1994c). On the other hand, antibodies to the IGF-1 receptor block keratinocyte growth induced by IGF-1 but do not inhibit basal proliferation (Varani et al., 1994c).

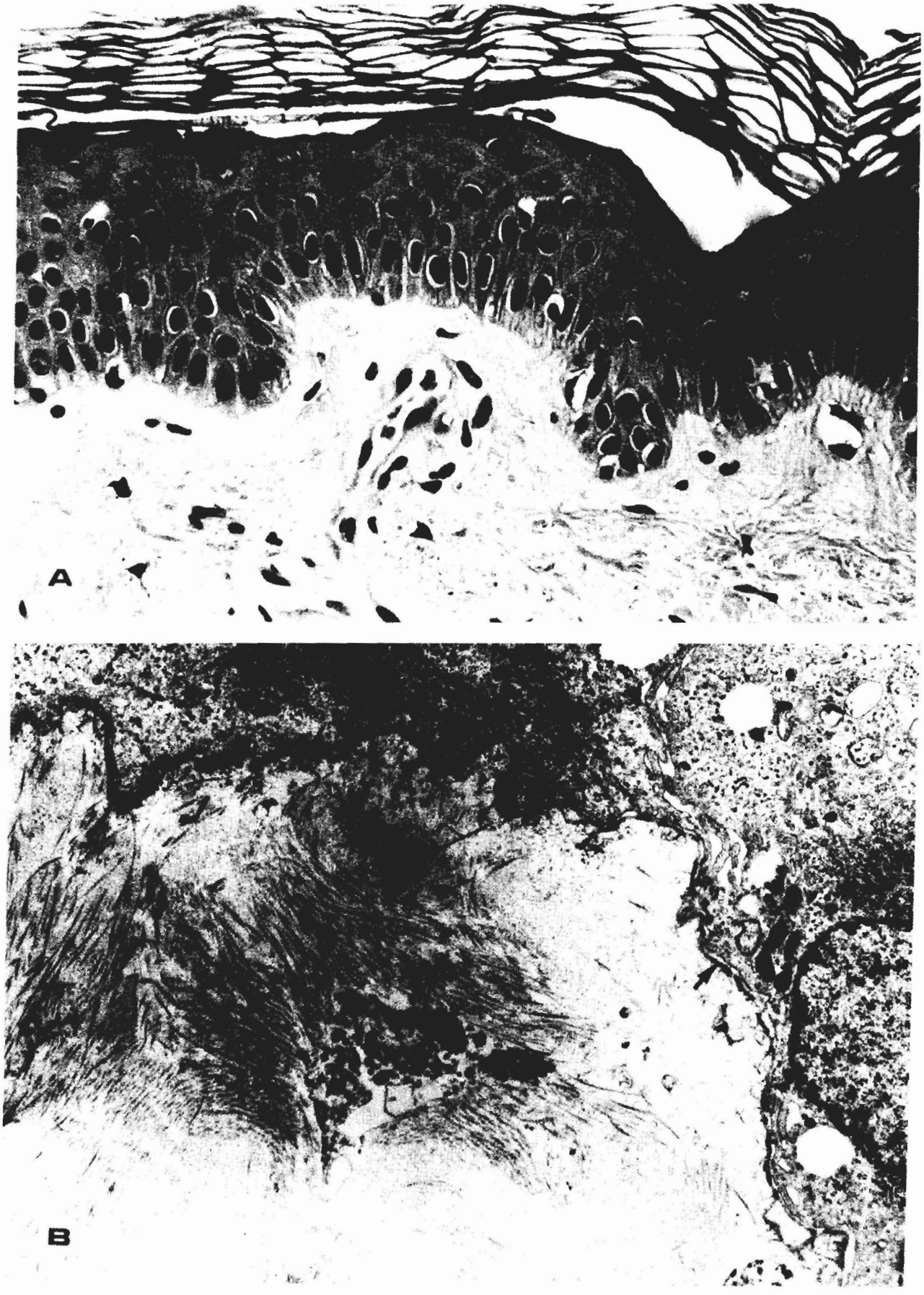
Human keratinocytes proliferate over a wide range of extracellular Ca^{2+} concentrations. At low Ca^{2+} concentrations (0.05-0.15 mM), the cells maintain an undifferentiated phenotype, while higher concentrations induce differentiation (Hennings et al., 1980; Boyce and Ham, 1983; Milstone, 1987). This "calcium switch" has been used to elucidate the cellular changes that occur in keratinocytes as they undergo a transition from undifferentiated growth to differentiation, and to identify factors that mediate these changes. It has been shown that under low-Ca^{2+} conditions, the undifferentiated epithelial cells synthesize and secrete large amounts of several ECM components, including laminin, type IV collagen, thrombospondin and fibronectin (Clark et al., 1985a,b; Nickoloff et al., 1988; Varani et al., 1988). The undifferentiated cells also express surface receptors for these matrix molecules and utilize their receptors to adhere to the basement membrane (Riser et al., 1990). As the cells differentiate, synthesis of ECM material is reduced and adhesive capacity diminishes (Clark et al., 1985a,b; Nickoloff et al., 1988; Varani et al., 1988). Concomitantly, as keratinocytes undergo the transition from undifferentiated to differentiated growth, there is induction of proteins associated with the differentiated state, including high-molecular weight keratins and the enzyme that cross links these proteins (keratinocyte specific transglutaminase) (Yuspa et al., 1980; Pillai and Bikle, 1991). The cell layer becomes 3-dimensional. The cells in the upper layers terminally differentiate, eventually becoming a mass of cross-linked, high-molecular weight keratins.

Dermal fibroblasts also proliferate *in vitro*, and many of the same factors that promote keratinocyte growth stimulate fibroblast proliferation as well (Kaji and Matsuo, 1983; Plisko and Gilchrest, 1983; Phillips et al., 1984; Colige et al., 1990). Unlike keratinocytes, however, fibroblast growth is strictly regulated by extracellular Ca^{2+}. Fibroblasts require an extracellular Ca^{2+} concentration above 1 mM for proliferation. At concentrations below 1 mM, growth is inhibited and at concentrations as low as 0.05-0.15 mM (optimum for keratinocyte growth), fibroblasts die (Boynton et al., 1974, 1977). While dermal fibroblasts are usually grown *in vitro* at 1.4 to 1.6 mM extracellular Ca^{2+}, some studies have shown that higher concentrations resulted in even better growth (Praeger and Cristofalo, 1980, 1986). This is especially true in aged cells. On the other hand, studies have shown that transformation of fibroblasts results in a loss of Ca^{2+} sensitivity (Boynton et al., 1977). Factors such as EGF and IGF-1, which induce a proliferative response in high-Ca^{2+} (i.e., above 1 mM) medium, do not ameliorate the Ca^{2+} requirement. However, platelet-derived growth factor (PDGF) may act, in part, by lowering the Ca^{2+} threshold required for fibroblast proliferation (Betsholtz and Westermark, 1984). The effects of growth factors and extracellular Ca^{2+} on keratinocyte and fibroblast growth are demonstrated in Figure 2.

Retinoid effects on skin cells in monolayer culture

Retinoids have potent growth-regulating activity in the skin. When these agents are applied to normal human or mouse skin, they induce a hyper-proliferative

Fig. 1. Histological and ultrastructural appearance of freshly-biopsied skin. **A.** Human skin stained with hematoxylin and eosin and examined by light microscopy. **B.** Human skin examined by transmission electron microscopy. Long arrows indicate type I collagen bundles. Short arrows indicate basement membrane. A, x 470; B, x 9,000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response in the epidermis (Ashton et al., 1984; Griffiths et al., 1993). They also activate dermal fibroblasts and stimulate extracellular matrix production by these cells (Schwartz et al., 1991). Of interest, the same retinoids that stimulate growth in a quiescent epithelium have the capacity to suppress abnormal epithelial proliferation in conditions such as psoriasis (Rusciani et al., 1981). Likewise, these agents can suppress outgrowth of epithelial cell tumors from pre-cancerous lesions (Verma et al., 1979).

Efforts have been made to understand these complex effects using skin cells in culture. Retinoid effects *in vitro* appear to be as complex as they are *in vivo*. All-trans retinoic acid (RA) stimulates keratinocyte proliferation when the cells are maintained in a culture medium devoid of other growth factors, but inhibits the growth of keratinocytes that are maintained in a growth factor-enriched medium and are already proliferating actively (Varani et al., 1989). Retinoids also inhibit keratinocyte differentiation, but this appears to be independent of other growth factors. Interestingly, ECM production and cell-substrate adhesion are also reduced in retinoid-treated epithelial cells (Varani et al.,1989).

RA can also either stimulate or inhibit dermal fibroblast growth, depending on the state of the cells at the time of treatment. The most striking effects of retinoids on fibroblasts are seen at low extracellular Ca^{2+} concentrations. Unlike growth factors such as EGF and IGF-1, retinoids overcome the block in proliferation resulting from low extracellular Ca^{2+} (Varani et al., 1990a). This is shown in Figure 3. In contrast to what is seen with keratinocytes, retinoids act synergistically with other growth factors in stimulating fibroblast proliferation. Retinoids also have complex effects on extracellular matrix production by fibroblasts. Collagen synthesis can be either inhibited or stimulated, depending on the cell source and state at the time of treatment. Collagen I synthesis is inhibited in rapidly-growing cells and this results from interference with

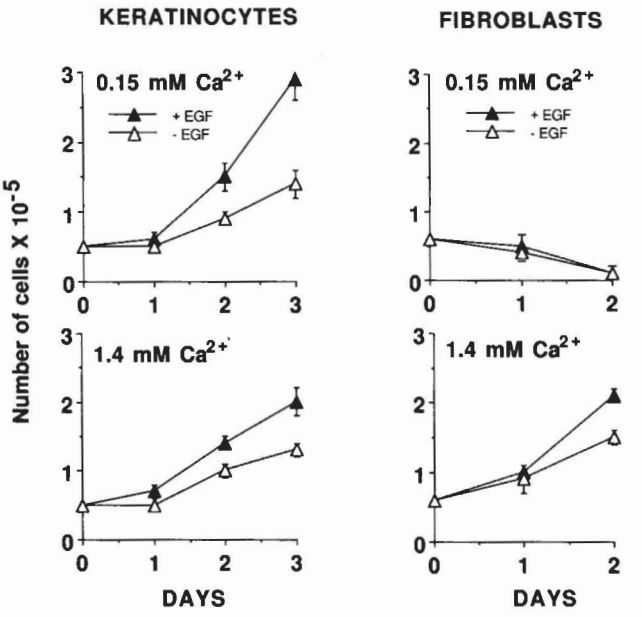

Fig. 2. Growth of human neonatal foreskin keratinocytes and fibroblasts in monolayer culture: effects of extracellular Ca^{2+} and EGF. Cells were plated at $5x10^4$ cells per well of a 24-well dish, and incubated for 48 hours in serum-free basal medium (Keratinocyte Basal Medium; Clonetics, San Diego, CA). The culture medium contained 0.15 mM or 1.4 mM extracellular Ca^{2+} and/or 1ng/ml human recombinant EGF as indicated. At the end of the incubation period, cells were harvested and counted.

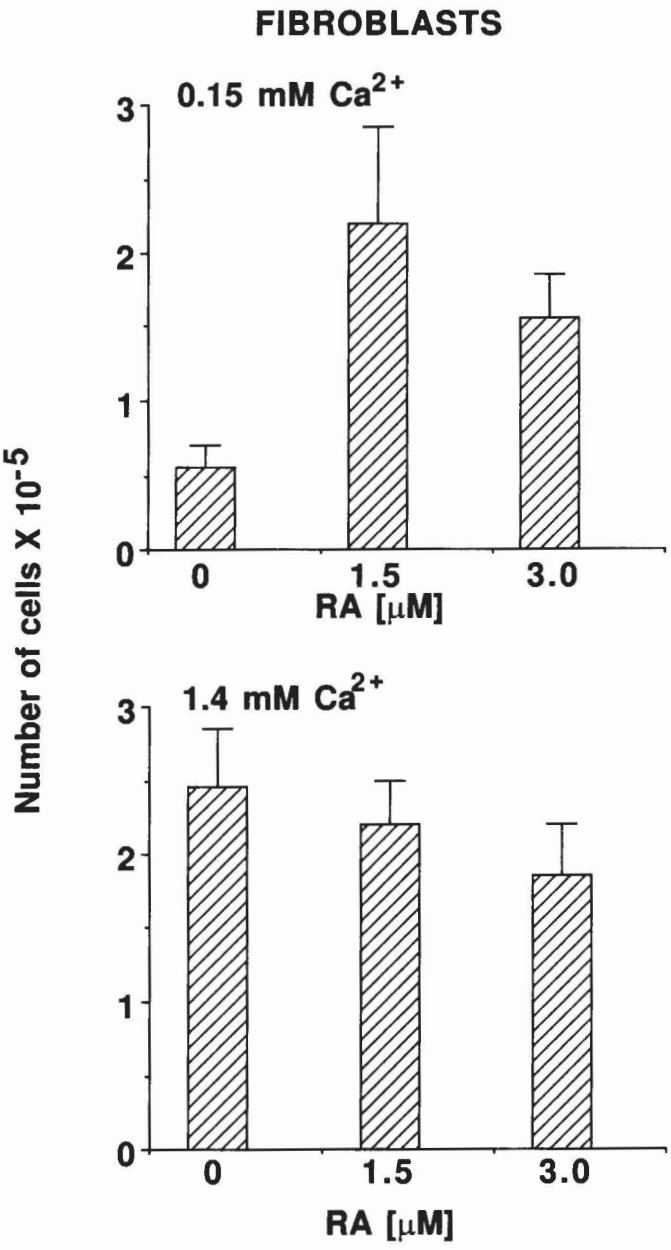

Fig. 3. Effects of RA on growth of human neonatal foreskin fibroblasts in monolayer culture. Cells were plated at $5x10^4$ per well of a 234-well dish. Cells were incubated for 48 hours in Keratinocyte Basal Medium supplemented with 1 ng/ml human recombinant EGF and with extracellular Ca^{2+} and/or RA as indicated. At the end of the incubation period, cells were harvested and counted.

procollagen I gene transcription (Meisler et al., 1997). In growth-inhibited cells, new collagen I synthesis occurs along with synthesis of other proteins (Varani et al., 1990b). Likewise, production of non-collagenous components of the extracellular matrix is stimulated as part of the growth response.

In summary, monolayer culture studies are useful for identifying conditions that promote keratinocyte and fibroblast survival and proliferation. They also indicate the growth factors that are capable of inducing a response, and provide an indication of other biological responses that occur in conjunction with proliferation. It is clear from these studies that how keratinocytes and fibroblasts respond to a given agent depends as much on the state of the cells at the time of treatment as it does on an innate capacity of the stimulating agent to induce a response. How the condition of keratinocytes and fibroblasts in intact skin relates to conditions seen in monolayer culture is not known, and it is difficult to known, therefore, the relevance of these *in vitro* findings to what occurs *in vivo*. Furthermore, cells grown in monolayer culture represent a sub-population of the cells present in the intact tissue. Thus, characteristic of culture-adapted cells may not be reflective of the properties expressed by the majority of cells originally present in the intact tissue. Because of these limitations, we have sought to use a more complex (organ) culture system to help elucidate the cellular and molecular basis for preservation of skin structure/function.

Maintenance of skin in organ culture; relationship between organ culture findings and results from monolayer culture studies

In the context of the skin, organ culture refers to small pieces of full-thickness skin (epidermis and dermis) incubated *in vitro* under conditions in which tissue histology and biochemical function are maintained but where cellular outgrowth from the tissue fragments does not occur. Practically, this involves incubating the tissue pieces either at an air-liquid interface or submerged in liquid culture medium over a support to which the tissue pieces do not adhere and which does not support outgrowth of cells. Approximately 2-mm sized pieces appear to be most satisfactory as larger tissue pieces show evidence of necrosis in the center after 5-6 days. Routinely, the tissue is incubated at 37 °C in an atmosphere of 95% air and 5% CO_2. In our laboratory, we provide a complete change of culture medium at 2-3 day intervals. Skin from virtually any site seems to be satisfactory, although there are age- and site-related differences in structure and function.

i) Conditions required for successful organ culture of skin

In an attempt to preserve tissue structure and function for extended periods of time in organ culture, we examined the same culture conditions that had been used successfully with keratinocytes and fibroblasts in monolayer culture. Specifically, 2-mm punch biopsies of adult human skin from a sun-protected site were incubated in a serum-free basal medium. The culture medium contained a level of extracellular Ca^{2+} (0.15 mM) that was optimized for keratinocyte growth in monolayer culture (Varani et al., 1989) or was supplemented with $CaCl_2$ to a final extracellular Ca^{2+} concentration of 1.4 mM (optimal for fibroblast growth, based on monolayer culture data (Varani et al., 1990a)). These media were used without change or further modified by the addition of one or more growth supplements. Among these were EGF, IGF-1, KGF, HGF, insulin, hydrocortisone or pituitary extract. All of these supplements facilitate keratinocyte growth in monolayer culture and many are also fibroblast growth factors. Figure 4 demonstrates the histological appearance of organ-cultured skin after incubation for 12 days. The major findings can be summarized as follows. Tissue architecture is maintained in growth factor-free, Ca^{2+}-supplemented medium (Fig. 4a,b), while complete necrosis is seen in growth factor-free, low Ca^{2+} medium (Fig. 4c). Growth factors do not alter the Ca^{2+} requirement; EGF is shown in Fig. 4d. Identical results were seen with the other factors and combinations of factors (see Varani et al., 1993a-c, 1994a,b for details of organ culture studies). The implication of these data is that culture conditions which maintain fibroblast viability and growth preserve tissue structure in organ culture, while conditions optimized for keratinocyte growth do not. As fibroblasts are a source of a number of keratinocyte growth factors, it seems reasonable to speculate that keratinocyte growth is mediated, at least in part, through paracrine loops involving dermal-derived growth factors. What specific dermal-derived factors are critical is yet to be determined. The only data to date bearing on this question suggest that the IGF-1 receptor on keratinocytes is important in maintaining epithelial integrity in organ culture (Varani et al., 1994c). By extension, this suggests that IGF-1 is an important dermal product.

Although Figure 4 presents results of histological evaluation only, a variety of other end-points support the same conclusion. In high-Ca^{2+} (fibroblast-optimized) medium, there is active synthesis of total proteins as well as components of the extracellular matrix (Varani et al., 1993a,b, 1994b). In low-Ca^{2+} medium, overall protein synthesis is much lower, and there is a large reduction in production of ECM components. Additionally, large numbers of viable keratinocytes and fibroblasts can be recovered from the skin after incubation in high-Ca^{2+} medium, but few cells are recovered from the low-Ca^{2+} cultures (Varani et al., 1994a,b). In addition to culturing whole skin in organ culture, experiments were conducted in which the dermis and epidermis were separated from each other and cultured separately. It was found in these studies that the isolated epidermis quickly degenerated while the isolated dermis remained viable and functionally intact for several days (Varani et al., 1994a).

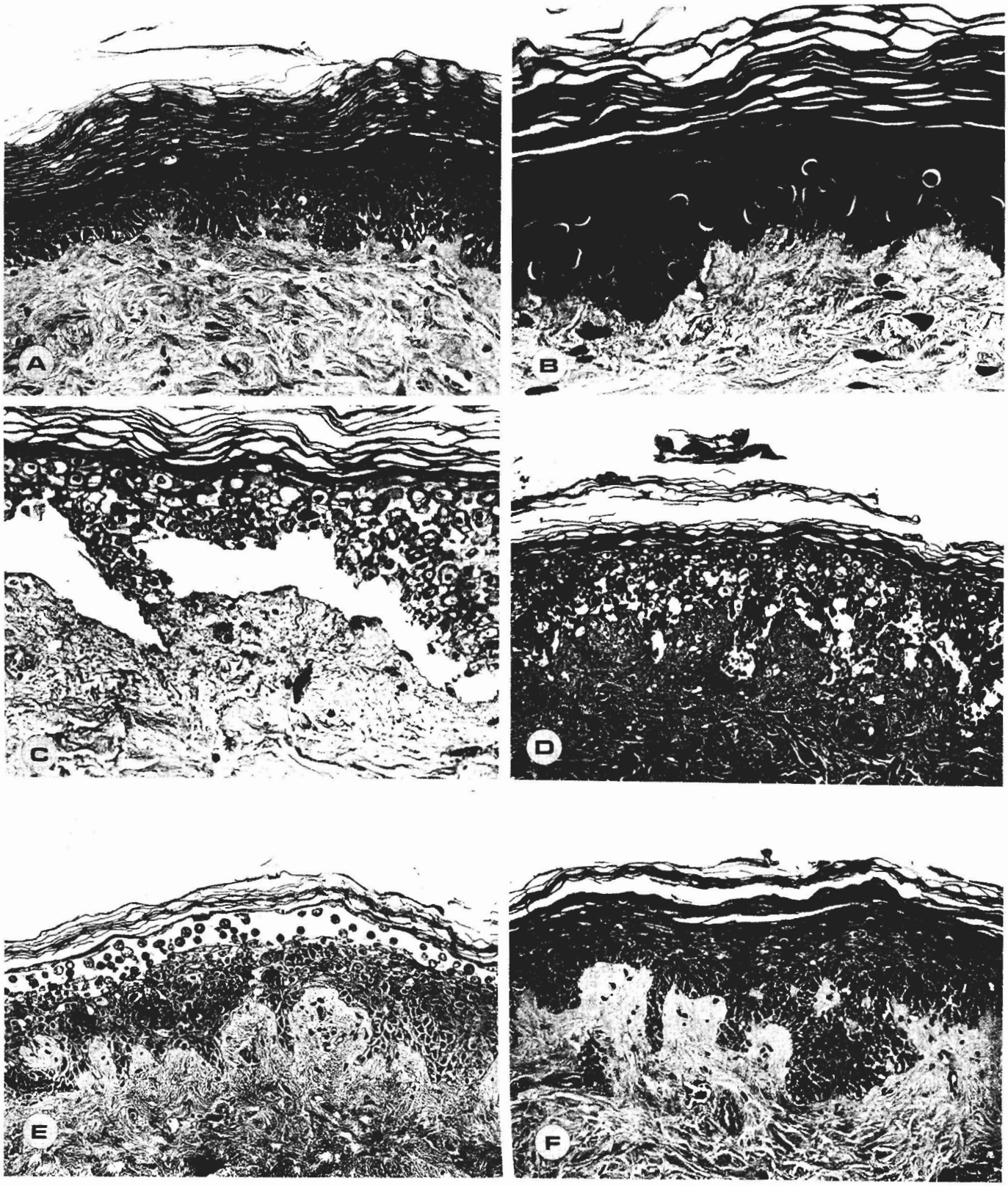

Fig. 4. Histological appearance of human skin after incubation for 12 days in organ culture. Two-mm punch biopsy cultures were incubated in serum-free Keratinocyte Basal Medium supplemented with Ca^{2+}, EGF or RA as indicated. At the end of the incubation period, the tissue was fixed in formalin, stained with hematoxylin and eosin and evaluated by light microscopy. **A.** 1.4 mM Ca^{2+} (x 175); **B.** 1.4 mM Ca^{2+} (x 360); **C.** 0.15 mM Ca^{2+} (x 360); **D.** 0.15 mM Ca^{2+} and 1 ng/ml EGF (x 180); **E.** 0.15 mM Ca^{2+} and 3 μm RA (x 125); **F.** 1.4 mM Ca^{2+} and 3 μm RA (x 125).

ii) Retinoid effects on human skin in organ culture: Further evidence for dermal role.

These observations imply that the dermis plays a key role in maintaining homeostasis in the skin. Results from studies with retinoids support this conclusion. As indicated above, RA is unlike growth factors such as EGF in that it promotes growth of human dermal fibroblasts in low-Ca^{2+} medium. Figure 4 shows the effects of RA on the histological appearance of skin after 12 days in organ culture under low-Ca^{2+} conditions. The dermis and lower layers of the epidermis are well-preserved, and there is no evidence of the necrosis in the basal epithelial cells that characterizes control skin.

A consistent feature of skin treated with RA under-low Ca^{2+} conditions is a failure of the epidermis to differentiate normally. Incomplete keratinization, acanthosis and sloughing of the upper epidermal layers is routinely seen. Why normal differentiation does not occur in RA-treated skin is not known. Retinoids are known to reduce epithelial cell cohesion and this has been shown both in monolayer culture studies (Varani et al., 1989, 1991) and *in vivo* (Williams and Elias, 1981). The histological features seen in RA-treated organ-cultured skin (Figure 4e) are consistent with a reduction in cohesion. Alternatively, extracellular Ca^{2+} is known to be a positive regulator of differentiation (Hennings et al., 1980; Boyce and Ham, 1983; Milstone, 1987; Pillai and Bikle, 1991) and it may be that the abnormal features seen in the upper epidermis reflect failure to differentiate in the low Ca^{2+} environment. This is not likely to be the complete story, however, since features of abnormal differentiation are also seen in organ cultures treated with RA under high-Ca^{2+} conditions (Fig. 4f), and reduced cohesion is also observed in some of the cultures.

It is clear from these studies that structure and function of human skin can be preserved for several days in organ culture. It is also clear that culture conditions optimized for dermal cell function are key. Dermal fibroblasts are likely to be important players, but other mesenchymal cells - e.g., dermal vascular endothelial cells - may also be critical. How dermal cells maintain viability of the epithelium is not fully understood. Fibroblasts and endothelial cells are a source of several keratinocyte growth factors and these may function in a paracrine loop. Likewise, dermal cells are a source of ECM components and some of these are incorporated into the epidermal basement membrane as well as the dermal connective tissue. While both growth factors and ECM components are likely to be important, we have been unsuccessful to date in finding a soluble dermal product or combination of products to replace the intact dermis itself. This undoubtably speaks to the overall complexity of the mechanisms operating to maintain the intact tissue.

Retinoid/Ca^{2+} effects on fibroblasts membrane function

The capacity of retinoids to modify the Ca^{2+} requirement has led us to investigate potential mechanisms by which this might occur. As an initial "working hypothesis" we speculated that retinoids might stimulate Ca^{2+} transport across the fibroblast plasma membrane and/or release intracellular stored Ca^{2+} into the cytoplasm. Either could help to maintain an adequate intracellular Ca^{2+} level in the face of low extracellular Ca^{2+}. Neither occurred. Using Ca^{2+}-sensitive fluorescent dyes as markers of intracellular free Ca^{2+}, it was found that treatment of fibroblasts with RA did not release stored Ca^{2+} (nor did it impair release triggered by ionomycin) and did not induce an influx of Ca^{2+} from the extracellular medium (Varani et al., 1995). However, it was found that RA interfered with the release of intracellular Ca^{2+} to the environment (Varani et al., 1995). Studies with $^{45}Ca^{2+}$-prelabeled cells, likewise showed that outward movement of Ca^{2+} was inhibited in retinoid-treated skin fibroblasts (Varani et al., 1990a). The ability of retinoids to sustain a sufficient level of intracellular Ca^{2+} by preventing its efflux could preserve viability by allowing Ca^{2+}-dependent process to be maintained.

How RA acts to prevent Ca^{2+} loss is not fully understood. It may be a direct membrane effect. This suggestion is based on the finding that membrane fluidity increased as the concentration of extracellular Ca^{2+} was reduced, and that RA, like Ca^{2+} itself, reduced fluidity (Varani et al., 1996). We speculate that under physiological conditions, interactions between surface receptors and their ligands lead to efficient signal transduction across the membrane, but that such signals are not effectively transduced across the expanded (fluidized) membrane that exists in the low-Ca^{2+} environment. By acting to reduce membrane fluidity, retinoids facilitate efficient transduction of growth-promoting signals. The implication of this is that the fibroblast plasma membrane is a critical target of retinoid action. This is not to negate the role of retinoids in gene transcription, but rather to suggest that membrane biophysical effects also occur. Of interest, retinoid concentrations that reduce membrane fluidity and prevent Ca^{2+} movement across the fibroblast plasma membrane are the same concentrations that promote fibroblast survival and growth under low-Ca^{2+} conditions and these are the same concentrations that preserve dermal and epidermal function in organ culture. Most importantly, studies by Duell et al. (1992) showed that the RA concentrations that are effective *in vitro* are the same concentrations found in the viable portion of the skin after topical application of a therapeutic doses of RA. This concentration is approximately 100-fold higher than the amount needed to activate retinoic acid receptors in the skin.

Use of skin organ cultures to investigate age-, site- and disease-related changes in skin

The underlying assumption of our studies is that elucidating how the structure and function of the skin are maintained under homeostatic conditions is essential to

understanding physiological processes such as ageing and wound repair; to understanding the pathophysiology of various disease processes; and to understanding mechanisms of response to toxic/therapeutic agents. While this assumption implies a hierarchy of effort, in reality, mechanisms of homeostasis (in normal skin) are probed in conjunction with efforts to understand how homeostasis is dis-regulated under various conditions. While discussion of such studies is beyond the scope of this review, it should be noted that organ culture technology has been used to probe mechanisms of such diverse diseases as psoriasis and pemphigus. Organ cultures of skin have also been used to probe mechanisms of action of topical drugs. In our own laboratory, we have used organ cultures of skin to probe question related to cancer invasion as well as the ageing process. The major advantage of organ-cultured skin for such studies is that it provides an intact tissue (similar to that found *in vivo*) for study, by yet the tissue can be manipulated as readily as cells in monolayer culture or other *in vitro* models.

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