0022-202X/83/8101S-0002s$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 81:2s–10s, 1983

Vol. 81, No. 1 Supplement
Printed in U.S.A.

REPORTS

The Reconstitution of Living Skin

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A living-skin equivalent useful as a skin replacement and as a model system for basic studies has been fabricated and tested extensively. It consists of two components: (1) a dermal equivalent made up of fibroblasts in a collagen matrix that is contracted and modified by the resident cells, and (2) an epidermis that develops from keratinocytes "plated" on the dermal equivalent.

A multilayered keratinizing epidermis with desmosomes, tonofilaments, and hemidesmosomes forms. Basement lamella formation occurs within 2 weeks in vitro when rat cells are used. With human cells, crypt or pseudofollicular morphogenesis is observed in vitro within 3 weeks after plating cells on the dermal equivalent.

Autografts and isografts of rat-skin equivalents made with cultured cells from biopsies are rapidly vascularized, block wound contraction, and persist essentially for the lifespan of the host. Seven to 9 days after grafting, donor cells become activated biosynthetically and mitotically. By 1 year, the dermal population decreases to a normal level and the matrix has been extensively remodeled. The grafts remain free of hair and sebaceous glands. Grafts to rats have been in place for over 2 years.

Now, allografts of dermal equivalents have been made across a major histocompatibility barrier and are not rejected. The persistence of cellular elements of the grafts is monitored by use of a genetic marker. Challenge of the allograft with a second skin-equivalent graft after 1 month does not result in rejection of the original graft or of the second skin-equivalent graft. We propose that allografts of tissue equivalents are tolerated because cells with class II antigens are selected against during in vitro cultivation and are excluded from the graft. Thus the fabrication of skin-equivalent tissues or of other equivalent tissues with parenchymal cells that do not bear class II antigens may render transplants of such tissues immunologically acceptable despite the presence of allogeneic cells. The capacity to graft across major histocompatibility barriers using living tissue equivalents may have important clinical significance.

The capacity to reassemble components of skin into a living functional tissue [1–3] has provided us with an opportunity to study aspects of the differentiation and function of keratinocytes and dermal fibroblasts in a defined organ model in vitro and in vivo and to formulate a new approach to skin transplantation and the problem of graft rejection. We present here a review of our work.

It has long been realized that tissue cells cultivated on plastic or glass may not function under the same conditions that prevail in vivo. This is especially true of connective-tissue cells, such as dermal fibroblasts, that ordinarily are surrounded by an extracellular matrix. However, it is also true of epidermal cells resident on a connective-tissue matrix that interact with the products of other cells, since such products diffuse through the matrix.

The matrix devised for the skin-equivalent model we have developed is initially relatively simple, but it is changed even in vitro as constituent dermal cells interact with it physically and contribute to it biosynthetically. It is also changed as keratinocytes attach to it, spread, and form an epidermis and basement membrane. Its form may be further modified as the epidermis, under certain conditions, undergoes morphogenesis.

To reconstitute a dermis, dermal fibroblasts are mixed with collagen, serum, and medium. In the right proportions, a gel forms (Fig. 1) and is contracted by the fibroblasts. The mixture can be poured into a mold whose shape determines the geometry of the contracted tissue. If poured into a petri plate, the tissue forms as a disk, of diameter much smaller than that of the plate. A fivefold decrease in diameter is usual. The contracted tissue equivalent floats just below the surface of the medium expressed from it. The rate of contraction is proportional to cell number and inversely proportional to lattice collagen content (Fig. 2). The condensation of fibrils of the lattice occurs as the result of a "collection" process executed by cells as they extend and withdraw cytoplasmic podia that attach to collagen fibrils. The latter are drawn toward the cell body in the course of podial contraction. Moving cells also pick up and carry with them some fibrils encountered during translocation. The active condensation of fibrils by cells releases or expresses fluid from the matrix, which further reduces interfibrillar distances. There is some degree of fibril ordering, since in the vicinity of cells, bundles of fibrils are aligned in parallel arrays (Fig. 3).

Grossly, the contracted dermal equivalent, after 1 to 2 weeks in vitro with or without an epidermal overlay, is tissue-like in its consistency (Fig. 4). It is slightly opaque and resists lengthwise stress without tearing. Cells in the dermal-equivalent matrix are responsive to their surrounds. After 4 to 7 days in the matrix in vitro, fibroblasts are growth-regulated; that is, they cease to incorporate DNA precursors [4] (Fig. 5). The regulation is not due to contact inhibition because cell density

This work was supported by Flow Laboratories and Grant No.

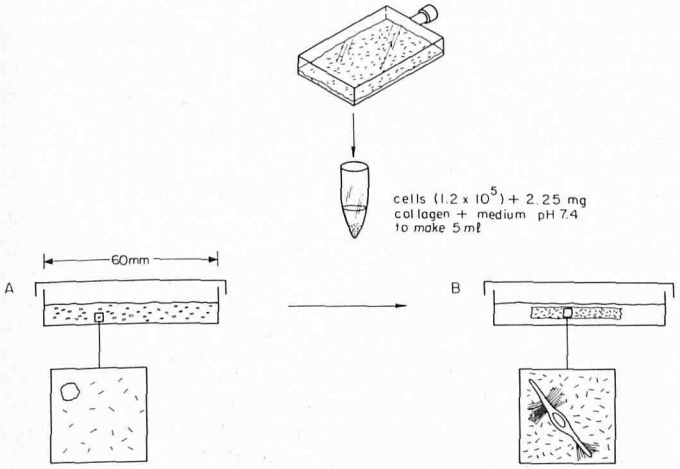


FIG 1. Procedure for forming a tissue equivalent: Cultivated cells are combined with collagen, medium, and 1.0 ml of fetal calf serum. Cells are added after the pH is raised to 7.4. *A*, The ingredients are quickly poured into a bacteriologic petri plate with swirling. Almost immediately the mixture gels. *B*, As cells compact collagen fibrils in a period of days, fluid is expressed from the collagen lattice that is gradually contracted away from the walls of the dish. It floats below the surface of the medium, which is squeezed out of the gel.

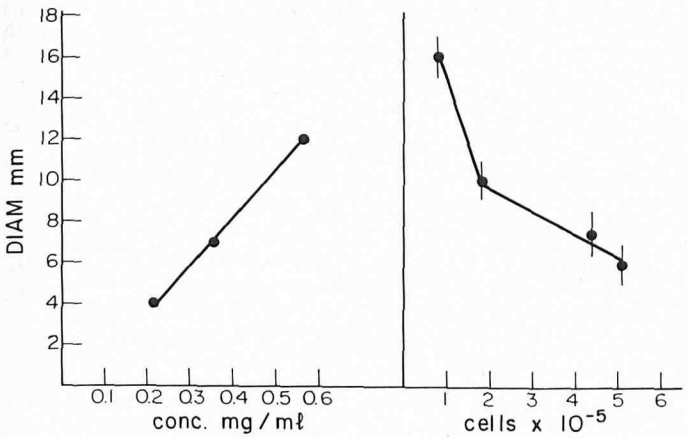


FIG 2. The extent of lattice contraction, measured as the diameter of the tissue equivalent attained after 10 days in vitro, is plotted as a function of collagen concentration (*left*) and cell number (*right*). Initial diameter of the uncontracted lattice is 53.0 mm. At the highest cell concentration shown, the lattice is fully contracted by 2 days [1].

Since cell cycling is blocked soon after cells are incorporated into the collagen matrix, the entire population becomes homogeneous with respect to DNA synthesis and can be compared with cells grown on monolayer, in which DNA synthesis is blocked because of contact inhibition. Such comparisons have been made with respect to several phenotypic features associated with the biosynthetic repertoire of dermal fibroblasts. Our results illustrate that cells in dermal-equivalent tissues are in a state of differentiation different from that of cells grown as a monolayer on plastic.

For example, perinuclear peroxidase activity is absent in human dermal log-phase cells and in cells grown to a confluent monolayer on plastic plates (Fig. 7). Furthermore, cells attached to the plastic substrate are impermeable to diaminobenzine, the reagent used to demonstrate oxidase activities. They become permeable to the reagent, however, when released from the substrate with trypsin. Entry of the reagent can be monitored

treatment is needed to make cells permeable to the reagent DAB [5] (Fig. 7).

Monolayered cells also differ from cells in dermal-equivalent lattices with respect to collagen processing. In cells in lattices,

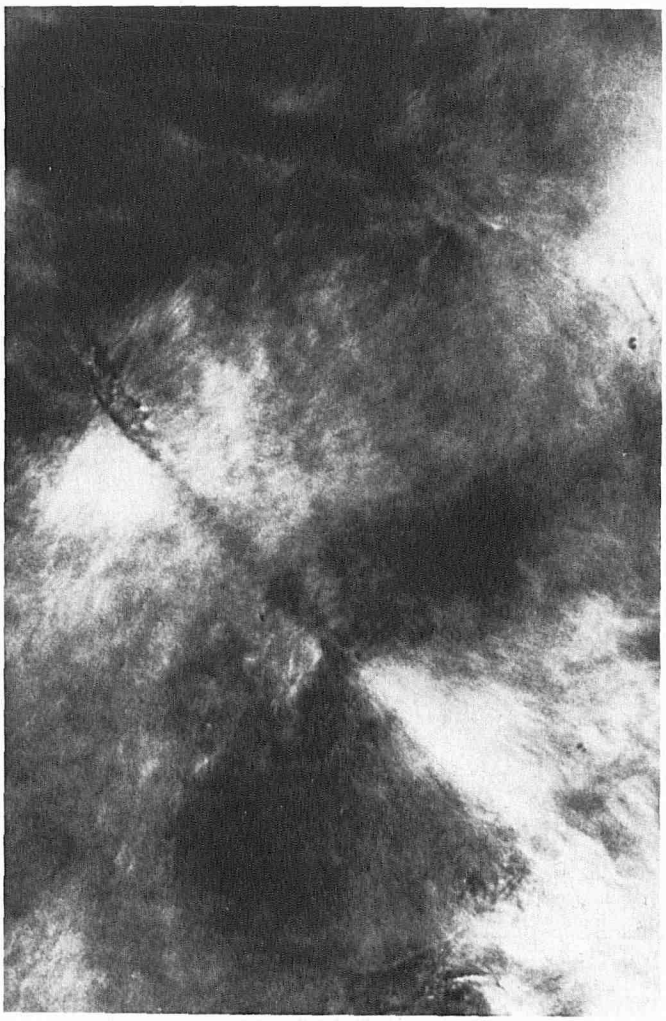
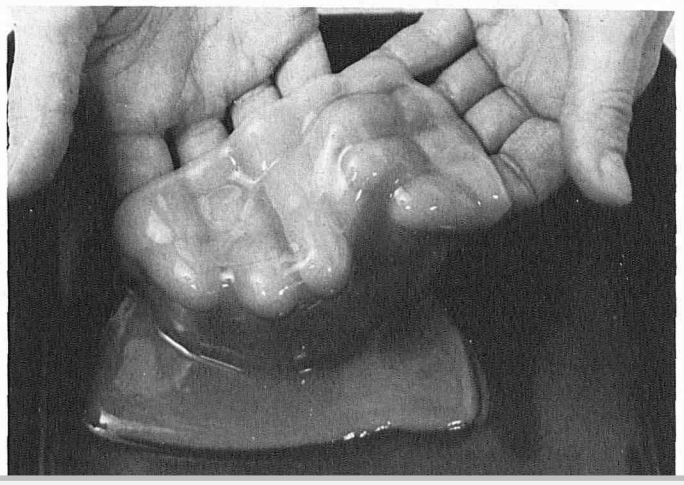


FIG 3. View of living fibroblasts in a tissue equivalent taken with a polarizing microscope. Fibrils of collagen are aligned in bundles closely associated with cells (×125).



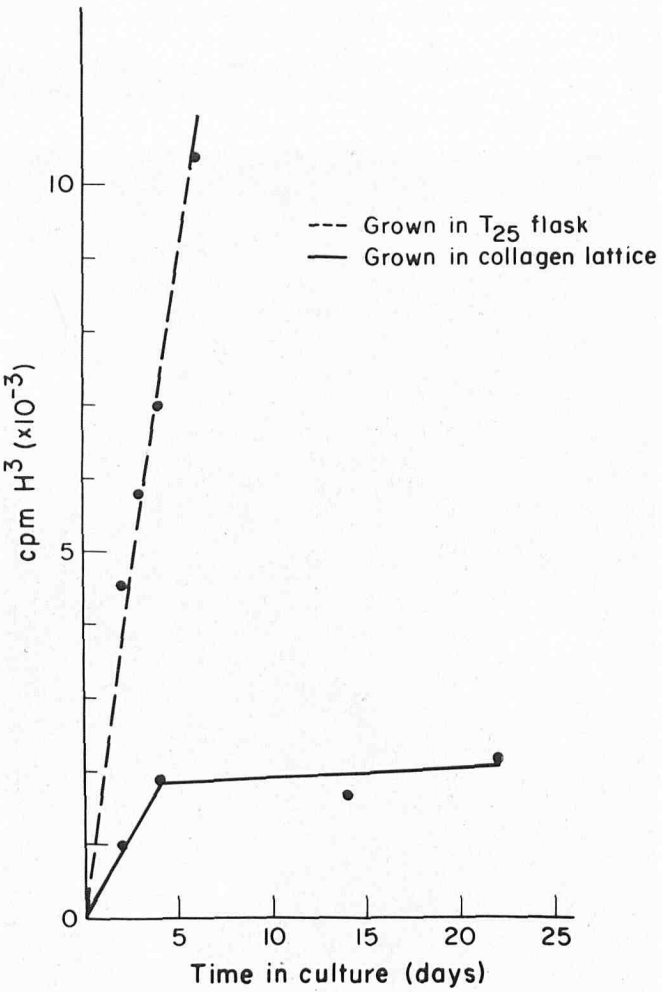


FIG 5. AG 1519 human fibroblasts (supplied by the Institute for Medical Research, Camden, N.J.) grown as monolayers or in tissue equivalents were labeled continuously with 1.0 μCi [methyl-^{3}H]-TdR. At each of the time points shown, samples were taken and acid-precipitable radioactivity measured after extracts were solubilized [4].

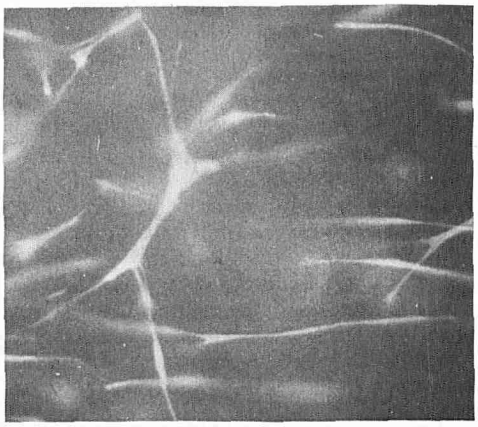


FIG 6. Cells incorporated into a dermal equivalent [4] and vitally stained with fluorescein diacetate after the tissue has contracted have ceased to incorporate DNA precursor even though they are well separated from one another (×1300).

regardless of population-doubling level, prolylhydroxylase ac-

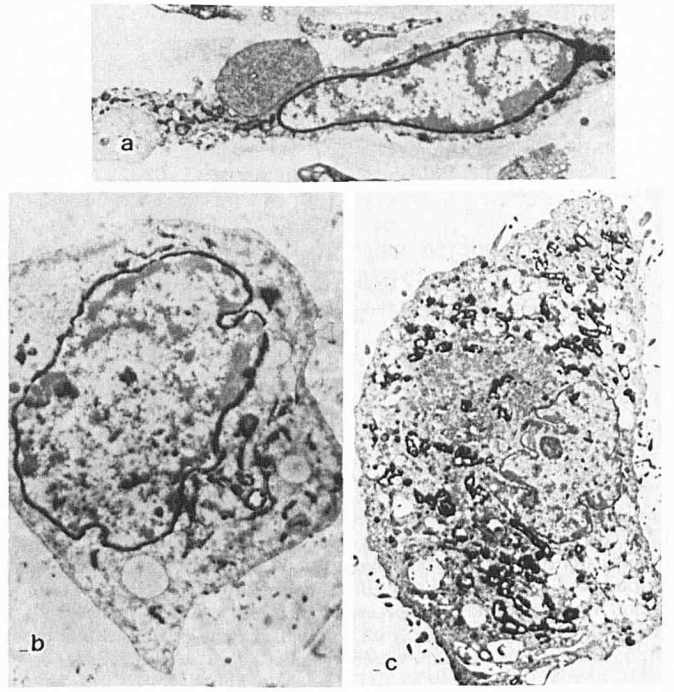


FIG 7. Human cells in a dermal equivalent for 3 days (*a*) and cells in a fresh biopsy (*b*) synthesize perinuclear peroxidase seen as a dark band around the nucleus after staining with diaminobenzine (DAB) [5]. *c*, Cells grown as monolayer show no perinuclear staining. Note that all cells show staining of mitochondrial associated cytochrome *c* oxidase, indicating that the reagent, DAB, has penetrated the cells (*a*: ×7400; *b*: ×6300; *c*: ×5700)

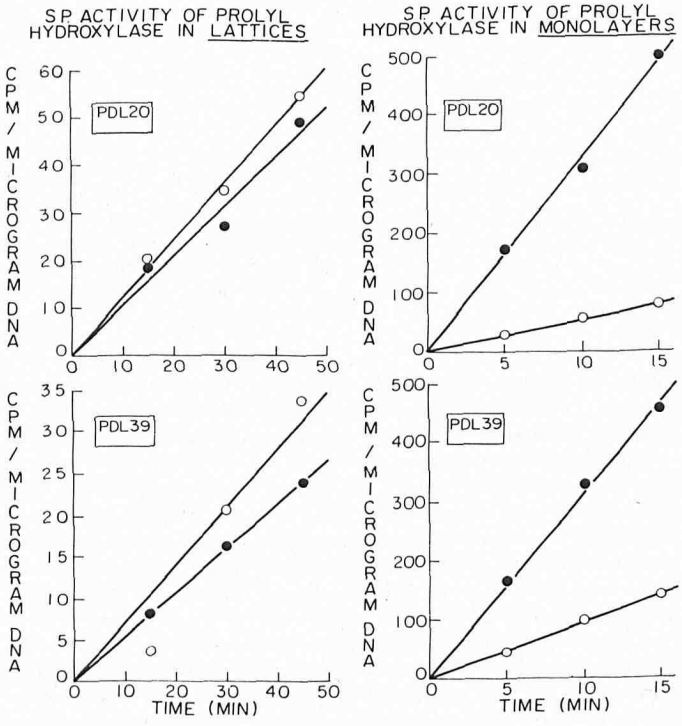


FIG 8. Effect of 0.2 m*M* ascorbate on the specific activity of prolyl-hydroxylase in cells of low (20 PDL) and high (39 PDL) population-doubling levels (PDL) grown in collagen lattices (*left*) or as monolayer

In addition, collagen is processed differently by cells grown in lattices, as compared with cells grown as a monolayer. While newly made collagen is added to the lattice structure of the dermal equivalent, in monolayer cultures, 95 percent of newly made collagen remains in solution unable to polymerize [7]. Other proteins synthesized by fibroblasts for export also become associated with the skin-equivalent matrix in vitro.

Several lines of evidence suggest that a lytic system able to degrade the dermal-equivalent tissue can become active in vitro [7]. First, chemical amounts of dialyzable labeled hydroxyproline can be found in the culture medium bathing the tissue equivalent; second, over a period of 9 days there is a measurable decrease in the unlabeled collagen used to make up the tissue equivalent; and third, degradation of newly synthesized collagen associated with the dermal equivalent is twice as great as degradation of newly made collagen in monolayer cultures. Given that there is both polymerization and deposition of newly made collagen in the tissue equivalent, as well as collagen degradation, the system provides an opportunity to study tissue remodeling in vitro.

Hence a number of enzyme systems, i.e., perinuclear peroxidase, prolylhydroxylase, a collagenolytic activity, and probably procollagen peptidases, behave differently in a tissue-equivalent milieu in vitro than in monolayer cell cultures. These findings suggest that cells in the tissue matrix in vitro are led to adopt a state of differentiation different from that of conventionally cultured cells. They also suggest that conditions in the tissue-equivalent model may be more like those which cells experience in vivo. This appears to be borne out in studies of more complex tissue constructs fabricated in our laboratory.

Human keratinocytes plated onto contracted dermal equivalents made up with human dermal fibroblasts quickly attach to the collagen substrate, multiply, and spread to form a continuous sheet [8]. The sheet differentiates, becoming multilayered with desmosomes, tonofilaments, and keratohyalin granules (Fig. 9). Rat epidermis on a dermal equivalent made up with rat fibroblasts has been shown to elaborate basement lamella in 2 weeks in vitro [21] (Fig. 10). With human epidermis, conditions that promote development of basement lamella have not yet been found. Even after 1 month in vitro, only hemidesmosomes have begun to differentiate. Specific antisera to collagen type IV have failed to give evidence of a lamellar structure. However, differentiation of keratin proteins by human keratinocytes grown on the dermal equivalent is quite normal in vitro, particularly when the epidermis is raised slightly above the level of the medium so that it is exposed to air. Protein extracts from labeled cells fractionated on SDS PAGE show the presence of low- but also high-molecular-weight keratins (65 K) (Fig. 11).

We report for the first time that human epidermis undergoes morphogenesis when cultivated for long periods on a dermal equivalent substrate in vitro [9]. By 3 weeks, deep crypts or follicle-like structures containing deposits of keratin are observed (Fig. 12*a*). The presence of fibroblasts is essential for crypt formation. Irradiation of fibroblast-contracted dermal equivalents with ^{60}Co prior to application of keratinocytes blocks crypt formation (Fig. 12*b*). When keratinocytes are incorporated into a collagen lattice with fibroblasts, they give evidence of intense biosynthetic activity when tissue equivalents are labeled with radioactive protein precursors (Fig. 12*c*). The absence of living fibroblasts results in a lowered level of biosynthetic activity (Fig. 12*d*). Epidermal differentiation also depends on the physical conditions of culture. When skin-equivalent tissues are cultivated below the surface of the medium, the epidermis constitutes itself as a monolayer and little crypt formation is observed; however, the epidermis becomes multilayered when exposed to a gaseous interface.

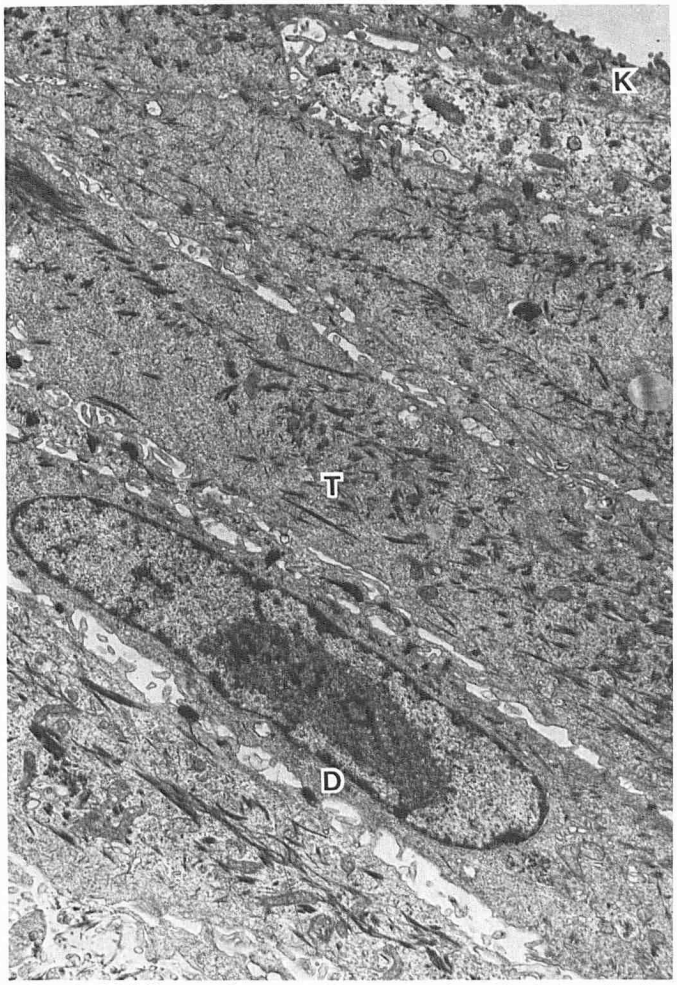


FIG 9. Multilayered human epidermis developed in 21 days in vitro after "plating" a suspension of keratinocytes obtained from a biopsy onto a contracted dermal equivalent. Desmosomes (*D*), keratohyalin granules (*K*), and tonofilaments (*T*) are apparent (reduced from ×8800).

when incorporated into a lattice, will contract it at a characteristic rate. The model has provided a way of comparing cells from old and young donors, cells of different population-doubling levels [1], or cells from different sources in the organism. The results can be precisely quantitated using change in tissue equivalent diameter to measure the rate of contraction. Cell number can be measured by determining DNA content fluorometrically [6]. The assay lends itself to study of the effects of environmental, pharmaceutical [20], or other agents that come into contact with the skin.

We wish to suggest that the model can be further elaborated (1) by manipulating the composition of the matrix and (2) by varying the character of the cell population. In work carried out so far, some cell types present in the dermis have been selected against, since the dermal equivalent has been constituted with cells passaged a number of times in vitro. For example, the deliberate inclusion of pigment cells has only recently been undertaken.

In summary, using skin proteins and skin cells derived from biopsies, an organ with many properties of skin has been reconstituted and used as a model system for studying biosynthesis, matrix modeling, differentiation, morphogenesis, and interactions among skin cells.

The physical resemblance of skin-equivalent tissues to skin

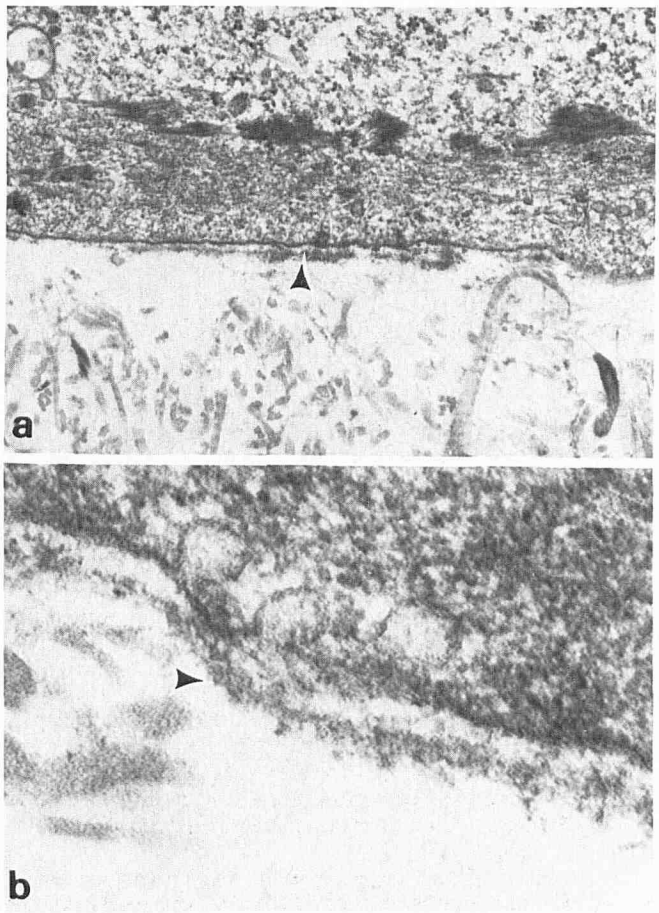


FIG 10. Basement lamella with hemidesmosomes lies between rat epidermis developed from a keratinocyte cell suspension and the contracted dermal equivalent containing rat dermal fibroblasts. The basement lamella (*arrows*) developed in vitro 2 weeks after keratinocytes were plated on the dermal equivalent (*a*: ×20,000; *b*: ×100,000).

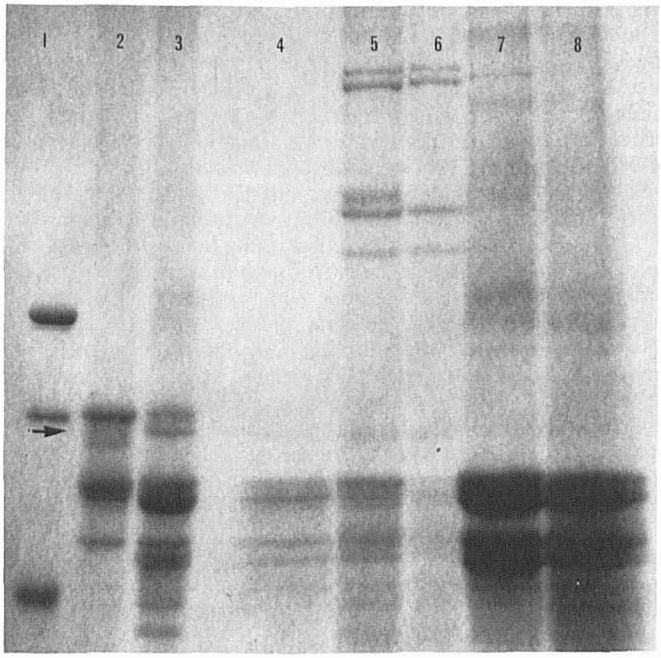


FIG 11. Autoradiogram of a polyacrylamide gel showing presence of a 65,000-dalton as well as lower-molecular-weight polypeptides. An extract of epidermis grown on a dermal equivalent for 21 days was layered on the slab gel. The tissue was labeled with 14 μCi/ml of an ^{3}H-amino acid mixture (New England Nuclear) for 72 hours prior to extraction. The molecular-weight standards were from Biorad; the middle band being serum albumin at 66,000 daltons. *Track 1*, standards; *2*, skin biopsy; *3*, primary epidermal culture on plastic; *4*, subculture on plastic; *5*, epidermis from submerged lattice; *6*, epidermis from unsubmerged lattice; *7*, epidermis applied to irradiated lattice submerged (gel overloaded); *8*, epidermis applied to irradiated lattice not submerged (gel overloaded). High-molecular-weight keratin bands are seen in tracks 2, 3, 5, and 6. Arrow points to ~65 K band.

After grafting, the skin equivalent heals within 7 days (Fig. 14). Eventually, the junction between graft and host becomes difficult to detect, as seen in a 5-month graft (Fig. 16*b*). The graft is initially translucent (Fig. 16*a*), making it possible to see the invading capillary circulation, but gradually it develops an opaque pink appearance, remaining soft and pliable. Grafts to rats at 1 month (Fig. 15) remain free of hair follicles and sebaceous glands, and their absence persists for the lifetime of the rat.

The cells of the dermal equivalent prior to transplantation appear to be less active biosynthetically than after transplantation. In fibroblasts of a 5-day graft, there is little rough endoplasmic reticulum, the majority of polysomes are free in the cytoplasm, and the nucleus is heterochromatic (Fig. 17*c*). Recall that the dermal fibroblasts are mitotically quiescent within a week after being cast in a lattice. By 5 days after grafting, cell density in the graft remains low, and cells in the immediate vicinity of invading capillary vessels are activated [10] (Fig. 17*a*). By 7 to 9 days after grafting, cell density increases dramatically [11] (Fig. 17*b*); virtually all fibroblasts of the dermal equivalent of the graft are activated and the graft as a whole is well-vascularized with a network of capillary vessels. Activation of the fibroblasts consists of elaboration of a well-developed rough endoplasmic reticulum, a decrease in the number of free polysomes, enlargement of the nucleolus,

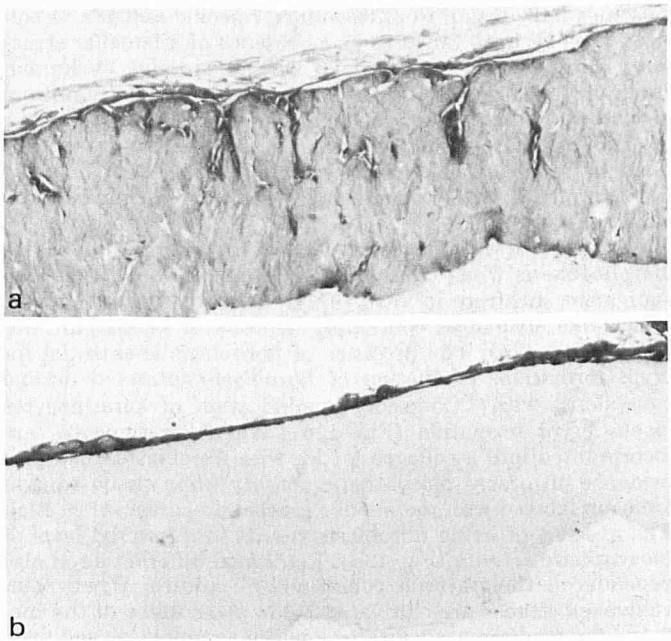


FIG 12. *a*, Crypts or follicle-like structures formed in vitro 3 weeks after a suspension of human keratinocytes is "plated" onto a dermal

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