Extracellular Matrix Proteins of Human Epidermal Keratinocytes and Feeder 3T3 Cells

KARI ALITALO, ESA KUISMANEN, RAILI MYLLYLÄ, URPO KIISTALA, SIRPA ASKO-SELJAVAARA, and ANTTI VAHERI
Department of Virology, University of Helsinki, Haartmaninkatu 3, 00290 Helsinki 29, Finland;
Department of Medical Biochemistry, University of Oulu, Kajaanintie 52 A, 90220 Oulu 22, Finland;
Department of Dermatology, Central Military Hospital, Mannerheimintie 164, 00280 Helsinki 28,
Finland; and Department of Plastic Surgery, Helsinki University Central Hospital, Kasarminkatu 11-13,
00130 Helsinki 13, Finland. Dr. Alitalo's present address is Department of Microbiology and
Immunology, University of California, San Francisco, CA 94143.

ABSTRACT   Cultures of human epidermal keratinocytes obtained from adult epidermis were initiated using irradiated BALB/3T3 cells as feeder layers. At different stages of confluence of the epidermal islands, feeder cells were removed and the extracellular matrix proteins of both pure component cells and cocultures were analyzed biochemically and by immunochemical methods and compared to those of skin fibroblasts of the same donors. The keratinocytes synthesized and secreted fibronectin and small amounts of laminin and type IV collagen. In addition, a nondisulfide-linked collagenous polypeptide ($M_r = 120,000$) was synthesized by the keratinocytes and was confined to the cell layers. Collagenous polypeptides with $M_r = 120,000$ were also synthesized by organ cultures of epidermal tissue and were detected in its acid or detergent extracts but again no secretion to culture medium was found. The $M_r = 120,000$ collagen had biochemical and immunological properties distinct from those of types I-V collagens. In immunofluorescence of keratinocyte cultures, fibronectin staining was prominent in the lining marginal cells of the expanding periphery of the epidermal cell islands but was not detected in the terminally differentiating cells in the upper layers of stratified colonies.

Very little type IV collagen was found deposited in pericellular matrix form by the keratinocytes. In contrast, the mouse 3T3 feeder cells were found to produce both type IV collagen and laminin in addition to the previously identified connective tissue glycoproteins of fibroblasts, interstitial procollagens, and fibronectin. Basement membrane collagen of the 3T3 cells was found deposited as apparently unprocessed procollagen $\alpha 1(IV)$ and $\alpha 2(IV)$ chains. The production in culture conditions of basal lamina glycoproteins by the fibroblastic feeder cells may promote the attachment and growth of the cocultured keratinocytes.

Epithelial cells in vivo rest on basal lamina structures known to promote epithelial differentiation (1–3) and to function in the remodeling of tissue type-specific structures (4). In culture conditions, the formation of basal lamina structure by epithelia has been shown to require contact with mesenchymal tissues (5–8), but the biosynthetic origin of specific basal lamina macromolecules such as type IV collagen, laminin, fibronectin, and proteoglycans, has been shown for only a few normal epithelia (9–12). We have here examined the capacity of cultures of human epidermal keratinocytes to synthesize proteins of basal laminae.

The epidermal-dermal junction has features unique among basement membranes (13, 14). It is composed of four distinct ultrastructures: (a) the basal cell plasma membrane with its specialized regions, (b) an electron-lucent area, the lamina rara, (c) the basal dense lamina, and (d) the subbasal lamina anchoring fibrils, dermal microfibril bundles, and collagen fibers.

During recent years, culture conditions have been developed to promote growth and differentiation of human epidermal keratinocytes (15, 16). The conditions supporting optimal growth of the keratinocytes as developed by Green and coworkers (16, 17) involve the use of 3T3 feeder cell layers and

epidermal growth factor and hydrocortisone as hormone supplements. The beneficial effect of feeder cells is not understood, but they can be removed after establishment of substrate-attached keratinocyte islands without an effect on keratinocyte growth. We now report that epidermal cells in these culture conditions produce fibronectin and synthesize and deposit small amounts of basement membrane collagen type IV and laminin as well as collagenous $M_r = 120,000$ polypeptides. Intracellular fibronectin is detected only in the lining marginal cells of the growing keratinocyte islands. The fibroblastic BALB/3T3 feeder cells secrete, in addition to interstitial procollagens and fibronectin, type IV procollagen and laminin that may also be needed for the substrate adhesion of the keratinocytes.

MATERIALS AND METHODS

Cell Cultures and Radioactive Labeling

Human epidermal cells were obtained from the roofs of artificially induced blisters suctioned in abdominal skin of adult volunteers as described (18, 19). The blistering method detaches epidermis along the lamina rara of the epidermal basal lamina (18, 20). The blister roof, essentially free of dermal elements (18–20), was cut off from freshly made blisters, washed in Eagle's minimal essential medium (MEM) containing penicillin (100 U/ml) and streptomycin (50 μg/ml), and treated with 0.25% trypsin for 30 min at 37°C. Cells were dispersed by pipetting in medium containing 10 μg/ml of DNase I (Merck, Darmstadt, FRG), washed, collected, counted, and seeded at a density of 5 × 10⁴cells/20 cm² together with one-third of confluent density of lethally irradiated (6000 rad γ-rays) fresh BALB/3T3 cells (clone A31, CCL 163, American Type Culture Collection, Rockville, MD) in MEM supplemented with antibiotics and 10% fetal calf serum (Gibco Bio-Cult, Glasgow, Scotland). In some experiments, epidermal tissue was separated with a forceps after collagenase (135 U/mg; Worthington Biochemical Corp., Freehold, NJ) treatment (2 mg/ml in MEM at 37°C for 2 h) from split-skin grafts taken with an electric dermatome, washed in collagenase-containing medium, and used either after dispersion of the cells or as organ cultures after washes in serum-containing medium. Beginning 2 or 3 d after inoculation, hydrocortisone (0.4 μg/ml), epidermal growth factor (EGF, 30 ng/ml; Collaborative Research Inc., Waltham, MA) and, for some cultures, cholera toxin (10⁻¹⁰ M, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY) were added (17). The cultures generally reached confluence in 4 wk. The cells were subcultured as described by Sun and Green (21), except that in some experiments a solution containing both trypsin (500 μg/ml) and EDTA (200 μg/ml) was used to selectively remove the 3T3 cells without detaching the keratinocytes. For control studies, primary and secondary cultures of human skin fibroblasts from the same donors, human tumor cell lines HT-1080 (ATCC CL121) and A431 (reference 22; gift from Dr. J. De Larco, Laboratory of Viral Carcinogenesis, National Institutes of Health, Bethesda, MD) producing exclusively type IV collagen (23, 24) and a human sarcoma cell line A204 producing exclusively type V collagen chains[1] as well as mouse parietal yolk sac carcinoma PYS-2 cells producing type IV collagen (25), were cultured without feeder cells.

For radioactive labeling of the keratinocytes, the 3T3 cells were first removed by repeated brief EDTA or trypsin-EDTA treatments and washed with serum-supplemented culture medium. As shown by Sun and Green (21), such treatment effectively and selectively removes feeder cells. Irradiated feeder cells also do not grow after the treatment. On the basis of counting of differentially distributed DNA in nuclei of stained human and mouse cells, we estimate the 3T3 cell contamination of pure keratinocytes to be <10 3T3 cells per 10⁶ keratinocytes. In control experiments, we used both untreated cultures (keratinocytes plus irradiated 3T3 cells) and irradiated and nonirradiated 3T3 cells. The cells were labeled with 5 μCi/ml of L-[U-¹⁴C]proline or with 20 μCi/ml of L-[5-³H]proline and/or [2-³H]glycine in the presence of 50 μg/ml of both sodium ascorbate and of β-aminopropionitrile fumarate (βapn) in regular medium or in medium lacking fetal calf serum but containing 0.05% bovine serum albumin. For labeling with high-specific activity [³⁵S]methionine, the cells were incubated in methionine-free medium for an hour before the addition of the isotope.

Analysis of Radiolabeled Proteins

Fibronectin was isolated from the culture medium by adsorption to gelatin-agarose beads (26). The presence of collagenous protein was tested by incubating

---

[1] Alitalo, K., R. Myllylä, H. Sage, P. Pritzl, A. Vaheri, and P. Bornstein. *J. Biol. Chem.* In press.

aliquots of culture medium with purified bacterial collagenase (30–50 U/ml for 60 min at 37°C; Advance Biofactures, Lynbrook, NY) or with buffer only. The collagenase and nonspecific protease activities in the digestions were controlled by including ¹⁴C-methylated type I collagen and bovine serum albumin (New England Nuclear, Boston, MA), respectively, into appropriate specimens. For digestion with pepsin (0.1 mg/ml, 24 h at 4°C), carrier collagen types IV and V were added, the pH of the sample was set to 3 with acetic acid, and the reaction was terminated by inhibition of pepsin activity with sodium hydroxide (to neutrality) and pepstatin A (1 μg/ml; Sigma Chemical Co., St. Louis, MO), after which the sample was dialyzed against neutral buffer and H₂O at 4°C and lyophilized.

Proteins were precipitated from the culture medium with ammonium sulphate (176 mg/ml) in the presence of protease inhibitors (24) or with 10% TCA at 0°C followed by washes with cold 5% TCA, 95% ethanol and absolute diethyl ether.

Cell layers were homogenized in 0.1 N acetic acid containing 1 μg/ml pepstatin A, dialyzed, clarified, lyophilized, and were digested with pepsin in 0.5 N acetic acid as the medium proteins were. For collagenase digestion and immunoprecipitations, the cell layers were harvested and samples were dialyzed in 1.2 M KCl/50 mM Tris/5 mM CaCl₂/1.2% Triton X-100, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride.

The polypeptides were analyzed by PAGE in the presence of SDS (27) followed by staining of the gels (28), fluorography (29) and, for some fluorographs, densitometric scanning. For determination of apparent molecular weights, nonradioactive globular protein markers (Pharmacia Fine Chemicals, Uppsala, Sweden), collagenous markers covalently prestained according to Griffith (30) or radioactive markers (Radiochemical Centre, Amersham, England) were used. Peptide mapping of polypeptide bands isolated by PAGE was carried out using *Staphylococcus V8* protease as described earlier (9).

Amino Acid Analysis

Both the peptides prepared by collagenase digestion and total dialyzed material from cultures or polypeptide bands from slab gels were assayed for protein-bound radioactive 3-hydroxyproline, 4-hydroxyproline, and proline in an amino acid analyzer after hydrolysis in 6 M HCl at 120°C for 16 h.

Immunological Procedures

The affinity-purified antibodies specific to procollagen types I and III and collagen types I, II, and IV (gift from Dr. Rupert Timpl (Max Planck Institute for Biochemistry, Federal Republic of Germany); reference 31) were used as described previously (9, 32). Antiserum against human plasma fibronectin was produced in rabbits (33) and was preabsorbed with fibronectin purified from fetal calf serum. Affinity-purified antikeratin antibodies were used as in another study (9; gift from Dr. I. Virtanen, University of Helsinki) on methanol-fixed cells. Double antibody immunoprecipitation from culture medium was carried out as described (32).

For immunofluorescence microscopy, the cell cultures grown on glass cover slips were rinsed three times with Pᵢ/NaCl and reacted with antibodies either in the cold (4°C), or after fixation with 3% paraformaldehyde (20 min) at room temperature for 20–30 min for staining of extracellular antigens. To expose intracellular structures as well, the cultures were treated with acetone (10 min at −20°C) or 0.05% Nonidet-P40 (NP-40) detergent (34). After three washes in Pᵢ/NaCl the cell layers were stained with commercially obtained (Wellcome, Beckenham, England) fluorescein isothiocyanate-conjugated goat antirabbit gamma-globulin at the recommended dilutions.

When needed for the labeling of DNA (35, 36), the benzimidazole derivative (Hoechst 33258) was added on the cover slips at a concentration of 0.5 μg/ml for 5 min before the final washes in Pᵢ/NaCl, distilled water and wet mounting. The mounting medium between the inverted cover slips and objective glass was 50% glycerol in 0.1 M Veronal-buffered saline (0.15 M, pH 8.6). Fluorescence was observed with a Leitz Dialux 20 microscope.

RESULTS

Identification of Extracellular Matrix Proteins Produced by Pure Cultures of Keratinocytes

Radioactive polypeptides were similar in media and cell layers of both growing and near-confluent pure keratinocyte cultures labeled for either 8 or 24 h, and in media labeled in the presence or absence of fetal calf serum, EGF or hydrocortisone. Amino acid analysis (Table I) showed that, in comparison to the donor fibroblasts and feeder cells, pure keratinocyte cultures had a low 4-hydroxyproline/proline ratio but a much higher 3-hydroxyproline/4-hydroxyproline ratio. The major

nondialyzable radiolabeled polypeptides seen in 5% polyacrylamide gels were precipitated by 30% ammonium sulphate, were distinctly different from those of the 3T3 cells, and had apparent mol wts (in the order of decreasing radioactivity incorporated from [³H]glycine) of 130,000, 150,000, 100,000, 120,000, 140,000, and 220,000 in reducing conditions (Fig. 1, lane 6; see also Fig. 3 B). In nonreducing conditions, all the major polypeptides migrated in disulfide-linked higher mol wt forms. The incorporation of amino acid label into protein, however, was greatly reduced in postconfluent, terminally differentiating keratinocyte cultures, freed of 3T3 cells.

The polypeptide at $M_r = 220,000$ was identified as fibronectin antigenically and by its affinity to gelatin-agarose (Fig. 1, lanes 1–6). No prominent radioactive polypeptides comigrating

FIGURE 2   (A) Analysis of [³H]glycine and [¹⁴C]proline-labeled (24 h) proteins in keratinocyte cell layers by SDS polyacrylamide gel electrophoresis and collagenase digestions. Extracts of cell layers (lanes 1–3) were incubated with (lane 2) or without (lanes 1 and 3) collagenase as described in Materials and Methods, dialyzed, lyophilized, and subjected to electrophoresis in reducing (HSEtOH+) and nonreducing (HSEtOH−) conditions. Lanes V and I refer to the marker samples of collagen type Vα1 and α2 chains from sarcoma cell cultures and α chains, β and γ components of type I collagen. (B) Staphylococcal protease V8 digestion of a mixture of purified unlabeled α1(V) chains from human amniotic membranes and the $M_r = 120,000$ collagenous [³H]-labeled polypeptide. 25 µg of α1(V) chains, 150,000 cpm of the $M_r = 120,000$ polypeptides, and 0.1 µg and 0.2 µg or 0.5 µg of V8 protease were used, as indicated. The reaction products were analyzed by electrophoresis in 12.5% polyacrylamide slab gel; the gel was stained for protein using Coomassie Brilliant Blue (CBB) and fluorographed (³H). The arrows indicate the origin separating gel and the front dye.
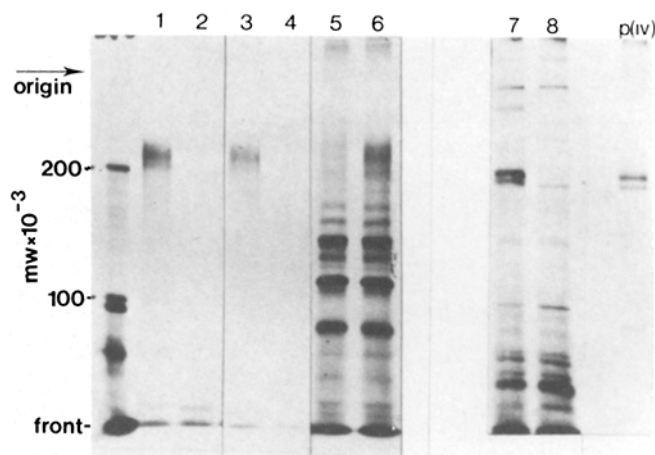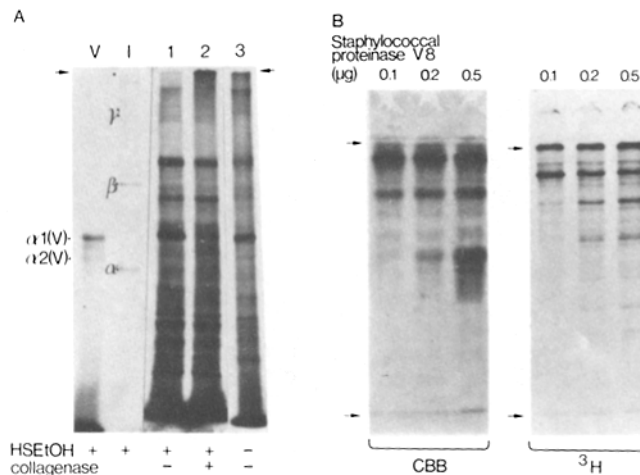
FIGURE 1   Isolation of fibronectin and type IV collagen from keratinocyte cultures. The [³H]glycine-labeled media were incubated with gelatin-Sepharose in (lane 2) or Sepharose 4B beads (lane 1) in the presence of protease inhibitors overnight at 4°C. The beads were collected by centrifugation, washed, and suspended in the electrophoresis sample buffer. Fibronectin polypeptides were also immunoprecipitated (lane 3). Lane 4 shows control precipitation with normal rabbit serum and lanes 5 and 6 the postimmunoprecipitation supernatants of samples 3 and 4, respectively. Immunoprecipitation of [³⁵S]methionine-labeled collagen type IV (lane 7) from the keratinocyte cell layers. Lane 8 shows material of lane 7 treated with purified bacterial collagenase and lane p(IV) the migration of [³H]glycine and [³H]proline-labeled procollagen type IV from the culture medium of HT-1080 cells. Analysis was made by electrophoresis in SDS polyacrylamide (5%) gels in reducing conditions. Noncollagenous marker proteins were run in the unnumbered lane on the left. mw, molecular weight.

with those of laminin were seen in the culture media of the keratinocytes, but they showed up in laminin immunoprecipitates from either glycine- and proline- or methionine-labeled cell layers (data not shown). Radiolabeled type IV procollagen was also immunoprecipitated from detergent extracts of the cell layers (Fig. 1, lanes 7 and 8). However, only faint labeling of type IV procollagen-sized polypeptides was observed in the media of cultures although labeling was done in the presence of ascorbate.

In addition to the procollagen type IV proα1(IV) and proα2(IV) polypeptides, collagenase digestion degraded some additional labeled polypeptide of $M_r = 85,000$ (mobility close to that of type I collagen α2(I) chains and in nonreducing conditions to that of γ-components) in the culture media of keratinocytes. The identity of this polypeptide was not clarified. Tests for the presence of interstitial procollagen types I and II as well as type II collagen by immunoprecipitation were negative. Amino acid analysis indicated that over half of radiolabeled hydroxyproline in keratinocyte cultures was insoluble in neutral buffer and nonionic detergent. Further collagens were therefore sought in the cell layers. In pepsin-digested material from the cell layers, some radioactivity comigrated with pepsin-resistant fragments of type IV collagen (24, 37), but no interstitial collagen chains were found. One major nondisulfide-linked collagenase-sensitive polypeptide ($M_r = 120,000$ using collagenous standards) was found in both acid and detergent extracts of the cell layers (Fig. 2A). This polypeptide comigrated close to the α1(V) chains of marker collagen but gave a one-dimensional peptide map distinct from that of type V collagen α1 chains (Fig. 2B). Hydroxyproline in the 120,000

collagenase-sensitive polypeptide of the keratinocyte cell layers confirmed its collagenous nature (data not shown).

Labeling experiments were also performed using organ cultures of isolated epidermal tissues. Labeled proteins secreted by the epidermis and those extracted with acid from it were analyzed for the presence of collagenous and noncollagenous proteins. No major collagenase-sensitive polypeptides were detected in the medium of organ cultures. In acid extracts of the tissue there was a major nondisulfide-linked polypeptide (Fig. 3A, lanes 2 and 6) that comigrated with the major collagenous polypeptide of the keratinocyte cell layers. It was also degraded by collagenase.

To compare the state of differentiation of the keratinocyte populations in cell and in tissue culture, their cytokeratin
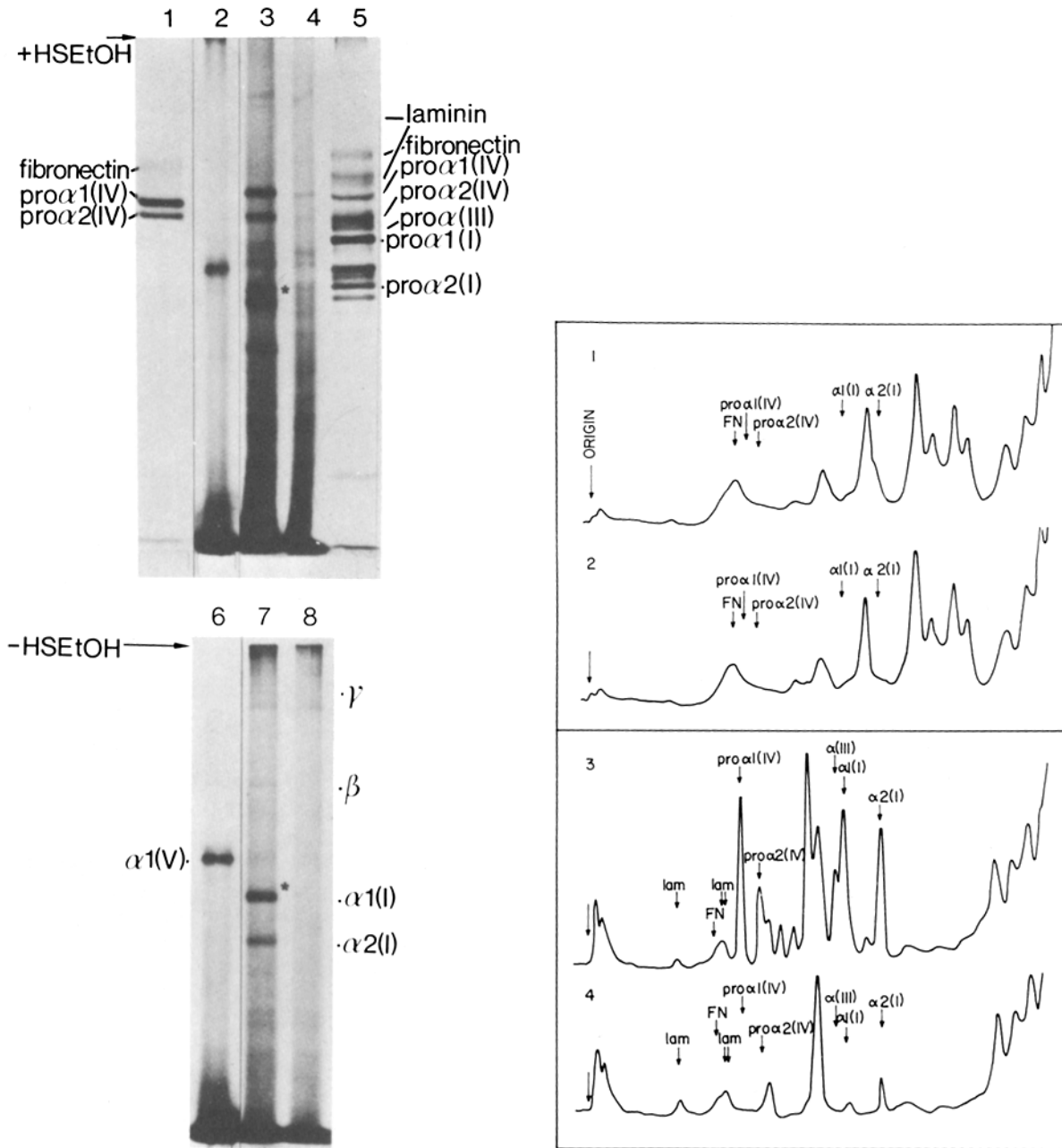

FIGURE 3   (A) Analysis of acetic acid-extracted proteins of labeled epidermis (lanes 2 and 6) and cell layers (lanes 3, 4, 7 and 8) and culture media (lane 5) of 3T3 cells. Samples of lanes 4 and 8 were collagenase-digested prior to electrophoresis. Lane 1 shows labeled proteins of the culture media of the HT-1080 cells, fibronectin and procollagen type IV chains. Note that human and mouse proα1(IV) and proα2(IV) chains do not comigrate (cf. references 24, 25, 40a). The identification of the polypeptides in the upper panel is based on their migration, sensitivity to collagenase, and immunoprecipitation. In addition, amino acid analysis of the procollagen type IV chains gave results typical for basement membrane collagen (not shown). The mobilities of marker α1(V), α1(I), and α2(I) chains are indicated in the lower panel. In the cell layer of the 3T3 cells, some type III collagen (mobility in reducing conditions indicated with an asterisk) is present, contrary to an earlier report (40). (B) Polyacrylamide (5%) gel electrophoresis in reducing conditions and densitometric scanning of autofluorograms of [³H]glycine- and [³H]proline-labeled proteins in culture media of the keratinocytes (lanes 1 and 2) and 3T3 cells (lanes 3 and 4) treated with collagenase (lanes 2 and 4) or with buffer only (lanes 1 and 3). The mobility of fibronectin (FN), laminin (lam), procollagen α1(IV) and α2(IV) chains (24), and standard α1 and α2 chains of collagen types I and III are marked.

polypeptides synthesized during the labeling experiments were compared, analyzed from the insoluble residues of the cell layers (cf. reference 38). The data, shown in Fig. 4, indicated that the cultures of epidermal keratinocytes consisted of cells representing various stages of differentiation.

Matrix Proteins Produced by Feeder Cells

In samples from 3T3 or human skin fibroblast cultures a major part of [^{3}H]glycine- and [^{3}H]proline-labeled radioactive polypeptides was degraded by collagenase (Fig. 3 A, lanes 3, 4, 7, and 8). Comparison of the labeled proteins in the culture media of the mouse 3T3 cells (Fig. 3 B, lanes 3 and 4) and human keratinocytes (Fig. 3 B, lanes 1 and 2) and donor skin fibroblasts (not shown) indicated that the proteins found in pure cultures of keratinocytes probably could not be derived from any contaminating fibroblastic cells.

The 3T3 feeder cells produced fibronectin and, in agreement with previous findings (39, 40), types I and III procollagen as their principal collagenous proteins (Fig. 3). In addition, 3T3 cells secreted procollagen type IV α1 and α2 chains and minor amounts of laminin into their growth medium. These matrix proteins were identified on the basis of their electrophoretic mobilities, collagenase digestion (Fig. 3), and immunoprecipitation (not shown). A distinct difference in the electrophoretic mobilities of human and mouse type IV procollagen chains (40a) was also noted. Thus the human origin of type IV collagen found in pure cultures of keratinocytes was further confirmed. The processing and deposition of interstitial procollagens was rapid especially in dense 3T3 cultures (see also reference 41), but basement membrane collagen was deposited considerably slower apparently in an uncleaved form (Fig. 3 A; compare lanes 3 and 5). The 3T3 cells also secreted laminin-related polypeptides with $M_r = 158,000$ that were immunoprecipitated with both our laminin-antiserum and a specific antiserum obtained from A. Cooper (Imperial Cancer Research Fund, Mill Hill Laboratories, London; reference 42; data not shown).

Immunoflourescence studies of keratinocyte cell layers confirmed the results of metabolic labeling and consistent results were also obtained in coculture conditions. Fibronectin was stained intracellularly in keratinocytes but notably only in cells close to the borders of the epithelial islands (Fig. 5 a and d) and at places pericellularly as faint matrix fibers under the cell islands. Some fibronectin staining was also seen in the 3T3 cells and it was prominent in their surrounding matrix fibers. In trypsin-EDTA-treated keratinocyte cultures, most pericellular staining was confined to the substratum beneath the keratinocytes (Fig. 5 g) and was visualized only at sites of their detachment. Antibodies to procollagen types I and III as well as to collagen type II showed no staining of the keratinocytes, and only minimal intracellular staining was seen with antibodies to mouse laminin and type IV collagen but intracellular staining of the 3T3 cells was obtained with all of these antibodies (for laminin and collagen type IV, see Fig. 5 b and c). The immunofluorescence results are summarized in Table II. DNA staining demonstrated mitoses in epithelial cell layers and also served to distinguish the stratified keratinocytes both from skin fibroblasts and from the irradiated 3T3 cells on the basis of their characteristic nuclear size and morphology (Fig. 5 f, k, l, and m).

Cytokeratin was detected in all human cells in the keratinocyte cultures but not in the 3T3 cells (Fig. 5 h and l). Positive staining of nuclei with human autoantisera (Fig. 5 j) indicated
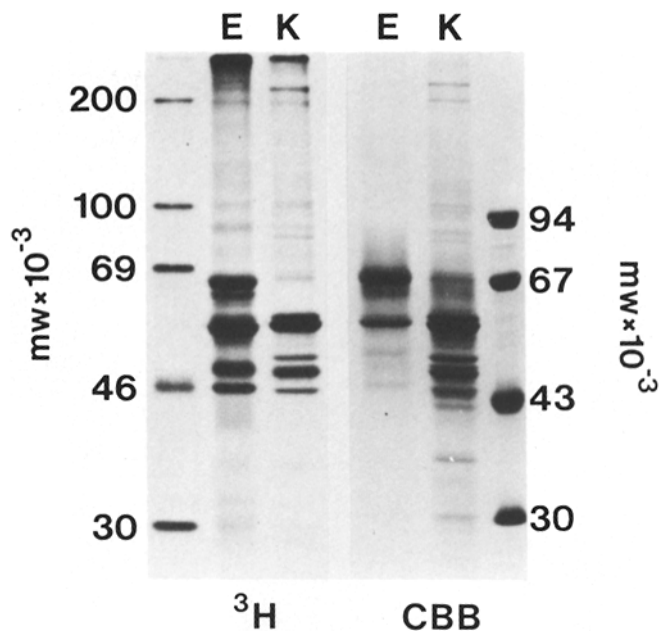

FIGURE 4   Polyacrylamide (8.5%) gel analysis, protein staining with Coomassie Brilliant Blue (CBB) and corresponding autofluorography (^{3}H) of acetic acid-insoluble keratins of epidermis (E) and of keratinocyte cultures (K) labeled with [^{3}H]proline and [^{3}H]glycine. Note that although the higher mol wt keratin polypeptides predominate in the epidermal tissue, small molecular weight keratin polypeptides characteristic of the basal layers of epidermis (38) are also labeled. Analysis of the samples in a 5% gel confirmed the absence of polypeptides comigrating with any of the chains of types I, III, or V collagens. Small amounts of two polypeptides comigrating with those of type IV procollagen were seen in the autofluorogram, but the hydroxyproline content of the sample was negligible. mw, mol wt.

that cells in detergent- or acetone-treated cultures, even the cells in the stratified cornifying layers of the central parts of the keratinocyte islands were indeed permeable to antibodies. The large cornified squames formed upon terminal differentiation of the keratinocytes (21) stained falsely positive for various antigens (Fig. 5 b and e), for reasons unknown.

DISCUSSION

What Proteins of the Epidermal-Dermal Junction Do Keratinocytes Produce In Culture?

We have here presented evidence that both fibronectin, type IV collagen, and laminin are produced in keratinocyte cultures. The latter two proteins were produced by all nonkeratinized cells, as judged by immunofluorescence. Results from amino acid analysis and immunoprecipitations indicate that only relatively small amounts of both laminin and type IV collagen are produced and that a major part of the two proteins is deposited in a poorly soluble form. Fibronectin was found also in the culture media but was produced only by some, as yet incompletely characterized, epidermal cells.

Keratinocyte cultures also synthesized collagenous proteins whose identity is still unknown. These were composed of the nondisulfide-linked $M_r = 120,000$ collagenous polypeptides that we found confined to keratinocyte cell layers and were also produced by epidermal tissue. A minor amount of $M_r = 85,000$ disulfide-linked collagenous polypeptides was found in the culture media.

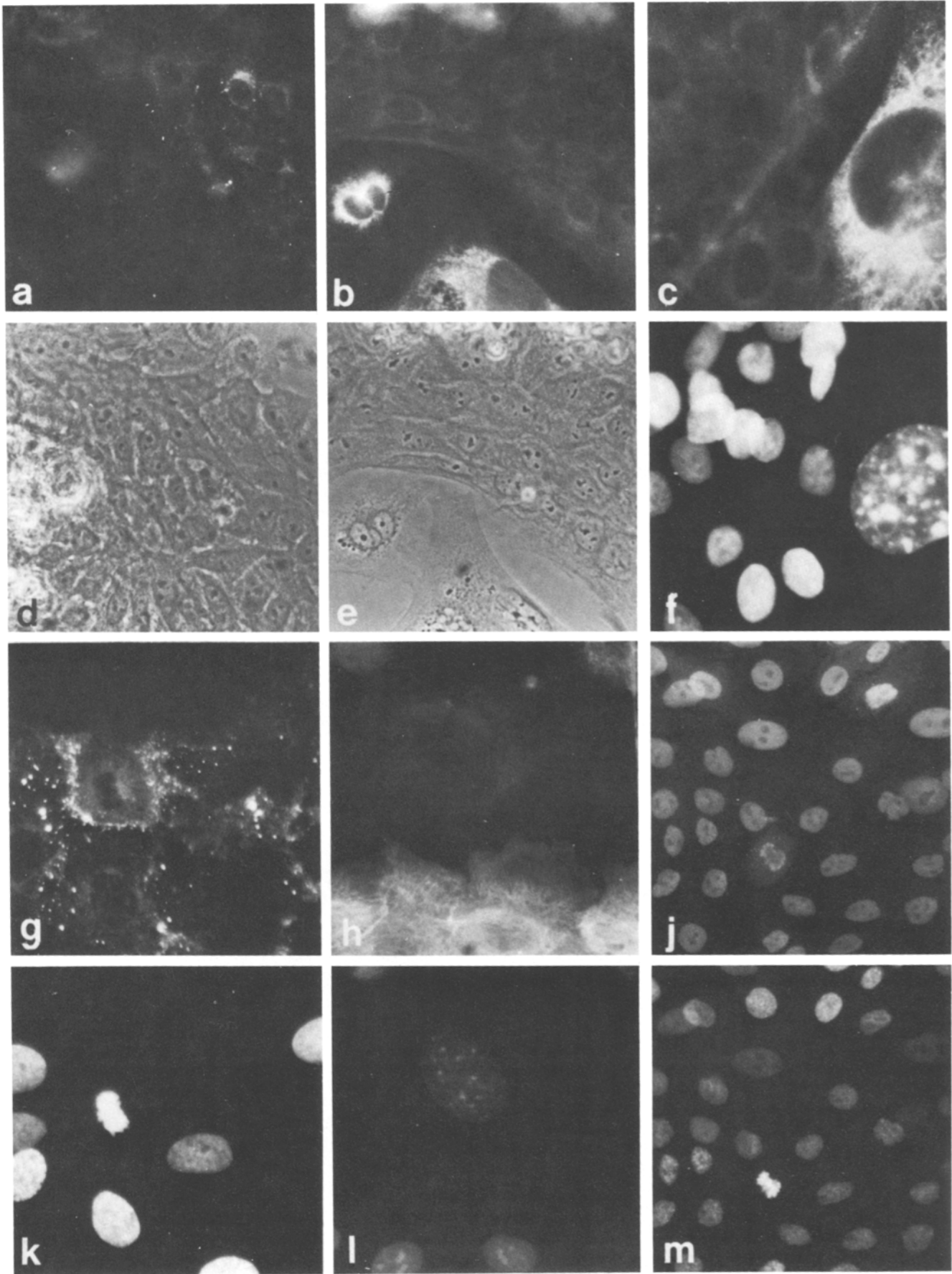Mesenchymal mouse BALB/3T3 feeder cells were also found

TABLE II

Summary of Immunofluorescence Analysis of Keratinocytes, 3T3 Cells, and Human Skin Fibroblasts

	Keratinocytes		3T3 cells		Donor skin fibroblasts	
	Intracellular	Pericellular	Intracellular	Pericellular	Intracellular	Pericellular
Fibronectin	+*	+	+	+++	+++	+++
Laminin	+	−	++	±	±	−
Procollagen type I	−	−	+++	+	+++	+++
Procollagen type III	−	−	+++	++	+++	+++
Collagen type IV	+	−	++	+	±	−
Cytokeratin	+++	−	−	−	−	−

* Only some cells were positive for fibronectin staining (see text for details).

to produce basal lamina glycoproteins, type IV procollagen, laminin, and M_r = 158,000 laminin-related polypeptides, in addition to the previously identified connective tissue matrix components of fibroblasts: interstitial procollagens and fibronectin.

Which Proteins of the Epidermal Basal Lamina Are Produced by Keratinocytes In Vivo?

The data published previously and here do not allow a conclusive answer. In vivo type IV collagen is detected in the lamina densa part of the epidermal basal lamina (43). Laminin (44) antigenicity is located in the lamina rara of skin basal laimina (20, 45) and it is thought to mediate the adhesion of epithelial cells to collagen type IV (46). Although many epithelial cells synthesize and secrete laminin, it is also produced by the fibroblastic 3T3 cells (reference 44 and this study).

Most cells, even epithelial ones, make collagen in culture (9, 47, 48). In tissue culture, the deposition of type V collagen was detected by immunofluorescence by Stenn et al. (49), and type IV collagen and laminin by Hintner et al. (8) at the epithelial outgrowths from skin explants (termed epiboly), but the biosynthetic origin of these basal lamina antigens was not determined. The present data show for the first time the capacity of keratinocytes to synthesize type IV collagen and laminin. Taken together, the data indicate a similar biosynthetic activity also in vivo.

The molecular identities of the collagenous M_r = 85,000 and 120,000 polypeptides are not yet known. The collagenous M_r = 85,000 polypeptide of the keratinocytes did, however, coprecipitate in some experiments with antibodies to type IV collagen. While its relationship to $\alpha1$ and $\alpha2$ chains of type IV collagen is being explored, it should be noted that several as yet poorly characterized polypeptides of type IV collagen may exist (50). When our studies were in process, Haralson et al. (51) reported on finding of homotrimer $[\alpha1(V)]_3$ collagen in pepsin-treated cell layers of a clone of Chinese hamster lung cells. Due to the apparent similarity of $\alpha1(V)$ chains of the M_r

= 120,000 polypeptides, we compared them by proteolytic peptide mapping. The results show the nonidentity of the two polypeptides. Also, no type IV collagen was found in pepsinized samples from hamster cell layers (51), and it is not known to us whether the hamster cells used by Haralson (51) are derived from defined epithelia. Other investigators have found that type V collagen may be produced by corneal keratinocytes (52). In its monomeric nature and apparent molecular weight, M_r = 120,000 collagenous polypeptide resembles a newly discovered type VI collagen (53, Alitalo, K., P. Bornstein, A. Vaheri, and H. Sage, manuscript submitted for publication).

Fibronectin is found between the lamina rara and the basal epidermal cell membrane (54). However, it may not be an intrinsic structural component of basal laminae and is in fact absent from normal glomerular basement membrane (36). Epithelial cells from many other sources, mostly from nonstratifying epithelia, produce fibronectin in culture (9, 55, 57). These include human amniotic (9), mammary (58), and endo- and ectocervical (59) epithelial cells. It was of interest to find intracellular fibronectin staining confined largely to the outermost cells of the keratinocyte islands. A centripetal gradient of differentiation is known to form in the growing colonies, with the least differentiated cells at the expanding periphery and terminally differentiating cells exfoliating on top of the colonies at their central parts (21). Thus, the synthesis of fibronectin may be lost early in keratinocyte differentiation. Another possibility is that the marginal cells are responding to culture conditions more like keratinocytes in re-epithelializing wounds. From the studies of Carlsson et al. (60) and Johansson et al. (61), it seems that both laminin and fibronectin may have distinct roles in mediating the adhesion of resting and migrating proliferating epithelial cells to extracellular substrata. Since all human cells in our cultures stained positively for keratin, we consider it unlikely that the fibronectin-positive cells would be derived from Langerhans cells or other contaminating cells in the keratinocyte cultures. The production of fibronectin by the epidermal cells or by 3T3 cells was seemingly not dependent on the presence of hormones reported to promote fibronectin

FIGURE 5 Indirect immunofluorescence (a, b, c, g, h, and j) DNA staining (f, k, l, and m) and phase contrast microscopy (d and e) of keratinocyte cultures stained with antiserum to fibronectin (a, d, g, and k), antibodies to laminin (b and e), collagen type IV (c and f), and keratin (h and l). To control the accessibility of antigens to antibodies in permeabilized cells used for staining in a, b, c, and h, the cell layers were also stained with human antinuclear autoantisera (gift from Dr. Marianne Gripenberg, University of Helsinki) in indirect immunofluorescence (j) and the result was compared with DNA staining of the same samples (m). Fig. 6 a depicts an area of the expanding periphery of a keratinocyte island. Note that only some cells are positive for intracellular fibronectin. 6 g and k show positive staining for pericellular fibronectin beneath a mitotic keratinocyte at the periphery of a keratinocyte island. Note the differential staining of the mouse 3T3 cells and human keratinocytes in b, c, and h, as distinguished by the corresponding phase-contrast image (e) or DNA-stained nuclei shown in f and l, respectively (3T3 cells have the large nuclei with coarsely aggregated chromatin). (a and d) × 150. (b, e, j, and m) × 250. (c, f, g, h, k, and l) × 400.

deposition in cultures of epithelial (62) and of malignant cells (63), and by the feeder layer 3T3 cells in serum-starved conditions (64).

The controversy about the phenotype of 3T3 cells (65, 66) was thought to be clarified by the results of Goldberg (39), who showed that the cells produce collagen types I and III characteristic of fibroblasts. Since then, it has been demonstrated that cells with a normal (human) fibroblastic phenotype can produce simultaneously procollagens of both basement membrane and interstitial types (32), and we now have detected procollagen type IV in cultures of pure clonal A31 3T3 mouse cells. The 3T3 cell line appears to be prone to phenotypic shift and drift according to culture conditions and in vivo (65, 67). In this respect, cells from genetically more stable species (68) may yield different results.

Is There a Function for the Matrix Components That the Feeder Cells Provide?

The production of basement membrane collagen and laminin by the 3T3 feeder cells may facilitate adhesion and spreading of the keratinocytes, since epithelial cells have been shown to adhere better to substrata coated with type IV collagen than to substrata coated with interstitial collagen types (69), probably through laminin (46). This cell-matrix interaction may also promote the effect of mitogens, as has been reported in other cell cultures (70), and thus explain the augmenting effect of medium conditioned by 3T3 cells on growth of the keratinocytes (17). The matrix proteins produced by mesenchymal feeder cells and hormones may also reduce the degradation of components of basal laminae produced by the epithelial cells, as has been reported for mammary epithelial cells (71, 72). Propagation for limited generations of human epidermal keratinocytes has been reported even in the absence of feeder layers of medium supplements other than serum (73, 74) and hydrocortisone (75, 76). It may be that in these conditions of dense plating the attachment of the keratinocytes to their growth substratum can occur through the matrix components they produce themselves.

In human keratinocyte cultures, and to a lesser extent also in cultures of squamous carcinoma cells, the failure to attach to the culture substratum will result in irreversible terminal differentiation (21, 77). Although several extracellular matrix proteins i.e., collagen type IV, laminin, fibronectin, and the M_r = 120,000 collagen, are produced by human epidermal keratinocytes, the relatively low amounts of laminin and collagen type IV synthesized may require supplementation from feeder cells for proper adhesion and growth of the keratinocytes.

epidermal basal keratinocytes. One population may represent stem cells while the other seems to anchor epidermis to the dermis. The relationship between such differentiated phenotypes and cells in keratinocyte cultures (e.g. cells producing fibronectin) remains to be studied.

REFERENCES

1. Grobstein, C. 1967. Mechanisms of organogenetic tissue interaction. *Natl. Cancer Inst. Monogr.* 26:279–299.
2. Hay, E. D. 1968. Role of basement membranes in development and differentiation. *In* Biology and Chemistry of Basement Membranes. N. A. Kefalides, editor. Academic Press, Inc., New York. 119–136.
3. Kefalides, N. A., R. Alper, and C. C. Clark. 1979. Biochemistry and metabolism of basement membranes. *Int. Rev. Cytol.* 61:167–228.
4. Vracko, R. 1974. Basal lamina scaffold-anatomy and significance for maintenance of orderly tissue structure. *Am. J. Pathol.* 77:314–338.
5. Briggaman, R. A., F. G. Dalldorf, and C. E. Wheeler. 1971. Formation and origin of basal lamina and anchoring fibrils in adult human skin. *J. Cell Biol.* 51:384–395.
6. Fusenig, N. E., D. Breikreutz, M. Lueder, P. Boucamp, J. Hornung, and P. K. M. Worst. 1980. Keratinization and structural organization in epidermal cell cultures. *Eur. J. Cell Biol.* 22:389a (Abstr.).
7. Hay, E. D., and J. W. Dodson. 1973. Secretion of collagen by corneal epithelium. I. Morphology of the collagenous products produced by isolated epithelia grown on frozen-killed lens. *J. Cell Biol.* 57:190–213.
8. Hintner, H., P. O. Fritsch, J.-M. Foidart, G. Stingl, G. Schuler, and S. I. Katz. 1980. Expression of basement membrane zone antigens at the dermo-epibolic junction in organ cultures of human skin. *J. Invest. Dermatol.* 74:200–204.
9. Alitalo, K., M. Kurkinen, A. Vaheri, T. Krieg, and R. Timpl. 1980. Extracellular matrix components synthesized by human amniotic epithelial cells in culture. *Cell.* 19:1053–1062.
10. Killen, P. S., and G. E. Striker. 1979. Human glomerular visceral epithelial cells synthesize a basal lamina collagen in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 76:3518–3522.
11. Linsenmayer, T. F., G. N. Smith, and E. D. Hay. 1977. Synthesis of two collagen types by embryonic chick corneal epithelium in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 74:30–43.
12. Quaroni, A., and R. L. Trelstad. 1980. Biochemical characterization of collagens synthesized by intestinal epithelial cell cultures. *J. Biol. Chem.* 255:8351–8361.
13. Briggaman, R. A., and C. E. Wheeler. 1975. The epidermal-dermal junction. *J. Invest. Dermatol.* 65:71–84.
14. Daroczy, J., J. Feldman, and K. Kiraly. 1977. Human epidermal basal lamina; its structure, connections and function. *In* Biochemistry and Pathology of Basement Membranes. A. M. Robert, R. Boniface, and L. R. Creteil, editors. S. Karger AG., Basel. 208–234.
15. Green, H., O. Kehinde, and J. Thomas. 1979. Growth of cultured human epidermal cells into the multiple epithelia suitable for grafting. *Proc. Natl. Acad. Sci. U. S. A.* 76:5665–5668.
16. Rheinwald, J. G., and H. Green. 1977. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature (Lond.).* 265:421–424.
17. Green, H. 1978. Cyclic AMP in relation to proliferation of the epidermal cell: a new view. *Cell.* 15:801–811.
18. Kiistala, U. 1968. Suction blister devide for separation of viable epidermis from dermis. *J. Invest. Dermatol.* 50:129–137.
19. Kiistala, U., and K. K. Mustakallio. 1967. Dermo-epidermal separation with suction. Electron microscopic and histochemical study of initial events of blistering on human skin. *J. Invest. Dermatol.* 48:466–477.
20. Saksela, O., K. Alitalo, U. Kiistala, and S. Vaheri. 1981. Basal lamina components in experimentally induced skin blisters. *J. Invest. Dermatol.* 77:283–286.
21. Sun, T.-T., and H. Green. 1976. Differentiation of the epidermal keratinocyte in cell culture: formation of the cornified envelope. *Cell.* 9:511–521.
22. Giard, D. J., S. A. Aaronson, G. J. Todaro, P. Arnstein, J. H. Kersey, H. Dosik, and W. P. Parks. 1973. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J. Natl. Cancer Inst.* 51:1417–1423.
23. Alitalo, K., J. Keski-Oja, and A. Vaheri. 1981. Extracellular matrix proteins characterize human tumor cell lines. *Int. J. Cancer.* 27:755–761.
24. Alitalo, K., A. Vaheri, T. Krieg, and R. Timpl. 1980. Biosynthesis of two subunits of type IV procollagen and of other basement membrane proteins by a human tumor cell line. *Eur. J. Biochem.* 109:247–255.
25. Leivo, I., K. Alitalo, L. Risteli, A. Vaheri, R. Timpl, and J. Wartiovaara. 1981. Basal lamina glycoproteins laminin and type IV collagen are assembled into a fine-fibered matrix in cultures of a teratoma-derived endodermal cell line. *Exp. Cell Res.* 137:15–23.
26. Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer.* 20:1–5.
27. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
28. Fairbanks, G., T. L. Steck, and D. F. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606–2617.
29. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritum-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83–88.
30. Griffith, I. P. 1971. Immediate visualization of proteins in dodecyl sulfate-polyacrylamide gels by prestaining with remazol dyes. *Anal. Biochem.* 46:402–412.
31. Timpl, R. 1982. Antibodies to collagens and procollagens. *Methods Enzymol.* 82:472–497.
32. Alitalo, K. 1980. Production of both interstitial and basement membrane procollagens by fibroblastic WI-38 cells from human embryonic lung. *Biochem. Biophys. Res. Commun.* 93:873–880.
33. Vuento, M., and A. Vaheri. 1979. Purification of fibronectin from human plasma by affinity chromatography under nondenaturing conditions. *Biochem. J.* 183:331–337.
34. Laurila, P., I. Virtanen, J. Wartiovaara, and S. Stenman. 1978. Fluorescent antibodies and lectins stain intracellular structures in fixed cells treated with nonionic detergent. *J. Histochem. Cytochem.* 26:251–257.
35. Russell, W. C., C. Newman, and D. H. Williamson. 1975. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. *Nature (Lond.).* 253:461–462.
36. Stenman, S., and A. Vaheri. 1978. Distribution of a major connective tissue protein, fibronectin, in normal human tissues. *J. Exp. Med.* 147:1054–1064.
37. Timpl, R., P. Bruckner, and P. Fietzek. 1979. Characterization of pepsin fragments of basement membrane collagen obtained from a mouse tumor. *Eur. J. Biochem.* 95:255–263.
38. Fuchs, E., and H. Green. 1980. Changes in keratin gene expression during terminal

differentiation of the keratinocyte. *Cell.* 19:1033–1042.

39. Goldberg, B. 1977. Collagen synthesis as a marker for cell type in mouse 3T3 lines. *Cell.* 11:169–172.
40. Goldberg, B. 1977. Kinetics of processing of type-1 and type-3 procollagens in fibroblast cultures. *Proc. Natl. Acad. Sci. U. S. A.* 74:3322–3325.
40a.Pihlajaniemi, T., R. Myllylä, K. Alitalo, A. Vaheri, and K. I. Kivirikko. 1981. Posttranslational modifications in the biosynthesis of type IV collagen by a human tumor cell line. *Biochemistry.* 20:7409–7415.
41. Taubman, M. B., and B. Goldberg. 1976. The processing of procollagen in cultures of human and mouse fibroblasts. *Arch. Biochem. Biophys.* 173:490–494.
42. Cooper, A. R., M. Kurkinen, A. Taylor, and B. L. M. Hogan. 1981. Studies on the biosynthesis of laminin by murine parietal endoderm cells. *Eur. J. Biochem.* 119:189–197.
43. Yaoita, H., J.-M. Foidart, and S. I. Katz. 1978. Localization of the collagenous component in skin basement membranes. *J. Invest. Dermatol.* 70:191–193.
44. Timpl, R., H. Rohde, P. Gehron-Robey, S. I. Rennard, J.-M. Foidart, and G. R. Martin. 1979. Laminin — glycoprotein from basement membranes. *J. Biol. Chem.* 254:9933–9937.
45. Foidart, J.-M., E. W. Bere, M. Yaar, S. I. Rennard, M. Guillino, G. R. Martin, and S. I. Katz. 1980. Distribution and immunoelectron microscopic localization of laminin, a noncollagenous basement membrane glycoprotein. *Lab. Invest.* 42:336–342.
46. Terranova, V. P., D. H. Rohrbach, and G. R. Martin. 1980. Role of laminin in the attachment of PAM 212 (epithelial) cells to basement membrane collagen. *Cell.* 22:719–726.
47. Green, H., and B. Goldberg. 1965. Synthesis of collagen by mammalian cell lines of fibroblastic and nonfibroblastic origin. *Proc. Natl. Acad. Sci. U. S. A.* 53:1360–1365.
48. Vaheri, A., and K. Alitalo. 1981. Pericellular matrix glycoproteins in cell differentiation and in malignant transformation. *In* Cellular Controls in Differentiation. C. W. Lloyd and D. A. Rees, editors. Academic Press, Inc., New York. 29–54.
49. Stenn, K. S., J. A. Madri, and F. J. Roll. 1979. Migrating epidermis produces AB₂ collagen and requires continual collagen synthesis for movement. *Nature (Lond.).* 277:229–232.
50. Timpl, R., and G. R. Martin. 1982. Components of basement membranes. *In* Immunochemistry of the Extracellular Matrix. vol. II. H. Furthmayr, editor. CRC Press, Boca Raton, Florida. In press.
51. Haralson, M. A., W. M. Mitchell, R. K. Rhodes, T. F. Kresina, S. Gay, and E. J. Miller. 1980. Chinese hamster lung cells synthesize and confine to the cellular domain a collagen composed solely of B chains. *Proc. Natl. Acad. Sci. U. S. A.* 77:5206–5210.
52. Sundar-Raj, C. V., C. Bowman, and J. Chao. 1981. In vitro tissue culture growth of human corneal and conjunctival epithelial cells and basement membrane glycoprotein syntheses. *J. Cell Biol.* 91 (2, Pt. 2):153a (Abstr.).
53. Sage, H., P. Pritzl, and P. Bornstein. 1980. A unique, pepsin-sensitive collagen synthesized by aortic endothelial cells in culture. *Biochemistry.* 19:5747–5755.
54. Couchman, J. R., W. T. Gibson, D. Thom, A. C. Weaver, D. A. Rees, and W. E. Parish. 1979. Fibronectin distribution in epithelial and associated tissues of the rat. *Arch. Dermatol. Res.* 266:295–310.
55. Chen, L. B., N. Maitland, H. Gallimore, and J. K. McDougall. 1977. Detection of the large external transformation-sensitive protein on some epithelial cells. *Exp. Cell Res.* 106:39–46.
56. Qaroni, A., K. J., Isselbacher, and E. Ruoslahti. 1978. Fibronectin synthesis by epithelial crypt cells of rat small intestine. *Proc. Natl. Acad. Sci. U. S. A.* 75:5548–5552.
57. Smith, H. S., J. L. Riggs, and M. W. Mosesson. 1979. Production of fibronectin by human epithelial cells in culture. *Cancer Res.* 39:4138–4144.
58. Taylor-Papadimitriou, J., J. Burchell, and J. Hurst. 1981. Production of fibronectin by normal and malignant human mammary epithelial cells. *Cancer Res.* 41:2491–2500.
59. Alitalo, K., H. Halila, E. Vesterinen, and A. Vaheri. 1982. Fibronectin production and keratinization in culture distinguish endo- and ecto- cervical human uterine epithelial cells. *Cancer Res.* 42:1142–1146.
60. Carlsson, E., E. Engvall, A. Freeman, and E. Ruoslahti. 1981. Laminin and fibronectin in cell adhesion: enhanced adhesion of cells from renegerating liver to laminin. *Proc. Natl. Acad. Sci. U. S. A.* 78:2403–2406.
61. Johansson, S., L. Kjellén, M. Höök, and R. Timpl. 1981. Substrate adhesion of rat hepatocytes: a comparison of laminin and fibronectin as attachment proteins. *J. Cell Biol.* 90:260–264.
62. Marceau, N., R. Coyette, J. P. Valet, and J. Deschenes. 1980. The effect of dexamethasone on formation of a fibronectin extracellular matrix by rat hepatocytes in vitro. *Exp. Cell Res.* 125:497–502.
63. Furcht, L. T., D. F. Mosher, G. Wendelschafer-Grabb, P. A. Woodbridge, and J. M. Foidart. 1979. Dexamethasone-induced accumulation of a fibronectin and collagen extracellular matrix in transformed human cells. *Nature (Lond.).* 277:393–395.
64. Chen, L. B., R. C. Gudor, T.-T. Sun, A. B. Chen, and M. W. Mosesson. 1977. Control of a cell surface major glycoprotein by epidermal growth factor. *Science (Wash. D. C.).* 197:776–778.
65. Boone, C. W. 1975. Malignant hemangioendotheliomas produced by subcutaneous inoculation of balb/3T3 cells attached to glass beads. *Science (Wash. D. C.).* 188:68–70.
66. Porter, K. R., G. J. Todaro, and V. Fonte. 1973. A scanning electron microscope study of surface features of viral and spontaneous transformants of mouse BALB/3T3 cells. *J. Cell Biol.* 59:633–642.
67. Bissel, M. J. 1981. The differentiated state of normal and malignant cells. *Int. Rev. Cytol.* 70:27–100.
68. Pontén, J. 1976. The relationship between in vitro transformation and tumor formation in vivo. *Biochim. Biophys. Acta.* 458:397–422.
69. Murray, J. C., G. Stingl, H. K. Kleinman, G. M. Martin, and S. I. Katz. 1979. Epidermal cells adhere preferentially to type IV basement membrane collagen. *J. Cell Biol.* 80:197–202.
70. Gospodarowicz, D., D. Delgado, and I. Vlodavsky. 1980. Permissive effect of the extracellular matrix on cell proliferation in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 77:4094–4098.
71. David, G., and M. Bernfield. 1981. Type I collagen reduces the degradation of basal lamina proteoglycan by mammary epithelial cells. *J. Cell Biol.* 91:281–286.
72. Salomon, D. S., L. A. Liotta, and W. R. Kidwell. 1981. Differential response to growth factor by rat mammary epithelium plated on different collagen substrata in serum-free medium. *Proc. Natl. Acad. Sci. U. S. A.* 78:382–386.
73. Eisinger, M., J. S. Lee, J. M. Hefton, Z. Darzynkiewicz, J. W. Chiao, and E. de Harven, 1979. Human epidermal cell cultures: growth and differentiation in the absence of dermal components or medium supplements. *Proc. Natl. Acad. Sci. U. S. A.* 76:5340–5344.
74. Hawley-Nelson, P., J. E. Sullivan, M. Krug, H. Hennings, and S. H. Yuspa. 1980. Optimized conditions for the growth of human epidermal cells in culture. *J. Invest. Dermatol.* 75:176–182.
75. Peehl, D. M., and R. G. Ham. 1980. Growth and differentiation of human keratinocytes without a feeder layer or conditioned medium. *In Vitro.* 16:516–525.
76. Peehl, D. M., and R. G. Ham. 1980. Clonal growth of human keratinocytes with small amounts of dialyzed serum. *In Vitro.* 16:526–538.
77. Rheinwald, J. G., and M. A. Beckett. 1980. Defective terminal differentiation in culture as a consistent and selectable character of malignant human keratinocytes. *Cell.* 22:629–632.
78. Alitalo, K., E. Kuismanen, and A. Vaheri. 1980. Basement membrane proteins synthesized by epithelial and neuronal cells in culture. *Eur. J. Cell Biol.* 22:432a (Abstr.).