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

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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 (Mr = 120,000) was synthesized by the keratinocytes and was confined to the cell layers. Collagenous polypeptides with Mr = 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 Mr = 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 a1(IV) and a2(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 co-workers (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 keratinoctye 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 keratinoctye islands. The fibroblastic BALB/3T3 feeder cells secrete, in addition to interstitial pro-collagens 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 detects 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 dispensed by pipetting in medium containing 10 μg/ml of DNase I (Merck, Darmstadt, FRG) washed, collected, counted, and seeded at a density of 5 × 10^5 cells/cm² together with one-third of confluent density of lethally irradiated (6000 rad) 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 Bin-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 mg/ml; Collaborative Research Inc., Waltham, MA) and, for some cultures, cholera toxin (10⁻⁷ 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 keratinoctyes. For immunological studies, primary and secondary cultures of human skin fibroblasts from the same donors, human tumor cells HT-1080 (ATCC CLI21) and A431 (gift from Dr. J. De Larco, Laboratory of Viral Carcinogenesis, Federal Republic of Germany); reference 31) were used as 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 P/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 P/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 P/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

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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 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 1 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 proalphaIV and proalpha2(IV) polyepitides, collagenase digestion degraded some additional labeled polypeptide of \( M_r = 85,000 \) (mobility close to that of type I collagen alpha1(I) chains) and in nonreducing conditions to that of \( \gamma \)-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 collagenases 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. 2 A). This polypeptide comigrated close to the \( \alpha_1(IV) \) chains of marker collagen but gave a one-dimensional peptide map distinct from that of type V collagen \( \alpha_1 \) chains (Fig. 2 B). Hydroxyproline in the 120,000

| Table 1 | Determination of the Proportion of Labeled Amino Acid Isomers in Keratinocyte Cultures |
|---------|------------------------------------------|
|          | 4-Hyro/pro | 3-Hyro/4-hyro |
| Cell cultures | I | II | I | II |
| KC          | 0.5 | 0.47 | 17.4 | 19.6 |
| OS          | 28.3 | 23.7 | 1.6 | 2.3 |
| 3T3         | 15.0 | 15.0 | 0.3 | 0.6 |
| 3T3 + KC    | 2.2 | 2.5 | 3.4 | 3.0 |

Pure cultures of keratinocytes (KC), of adult human skin fibroblasts (OS), of feeder cells (3T3), or feeder cell-supplemented keratinocyte cultures (3T3 + KC) were labeled for 24 h with radioactive amino acids in the presence of ascorbate and \( \beta \)-aminopropionitrile fumarate. Total cultures were then subjected to analysis of labeled amino acids in proteins. Results of two separate experiments (I and II) are given.
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

![Image](image_url)
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 [3H]glycine- and [3H]proline-labeled radioactive polypeptides was degraded by collagenase (Fig. 3 A, lanes 3, 4, 5, and 6). 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).

Immunofluorescence 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 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...
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 lamina (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 Strinn 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 \( \alpha_1 \) and \( \alpha_2 \) 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 \( [\alpha_1(V)]_3 \) collagen in pepsin-treated cell layers of a clone of Chinese hamster lung cells. Due to the apparent similarity of \( \alpha_1(V) \) 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 non-stratifying 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 production.

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).

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

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|---------------|-----------|------------------------|
| Intracellular | Pericellular | Intracellular | Pericellular | Intracellular | Pericellular |
| Fibronectin | ++ | + | + | +++ | ++ | ++ |
| Laminin | + | - | + | ++ | ± | - |
| Procollagen type I | - | - | +++ | + | +++ | +++ |
| Procollagen type III | - | - | +++ | + | +++ | +++ |
| Collagen type IV | + | - | +++ | + | ± | - |
| Cytokeratin | ++++ | - | - | - | - | - |

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 antiseraum 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 autoantiserum (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. 6a depicts an area of the expanding periphery of a keratinocyte island. Note that only some cells are positive for intracellular fibronectin. 6g 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 of 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, c, g, h, 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 basement lamina 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 proteins i.e., collagen type IV, laminin, fibronectin, and the Mr = 120,000 collagen, are produced by human epidermal keratinocytes, the relatively low amounts of laminin and collagen type IV in cultures of pure clonal A31 3T3 mouse cells. 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.

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