Journal of Cellular Biochemistry 28:127–141 (1985)
Extracellular Matrix: Structure and Function 247–261

Human Keratinocytes That Have not Terminally Differentiated Synthesize Laminin and Fibronectin but Deposit Only Fibronectin in the Pericellular Matrix

Richard A.F. Clark, Larry D. Nielsen, Samuel E. Howell, and Joy M. Folkvord

National Jewish Hospital and Research Center, Denver, Colorado 80206

Fibronectin and laminin production by human keratinocytes cultured in serum-free, low-calcium medium without a fibroblast feeder layer were examined by several techniques. By indirect immunofluorescence, fibronectin but not laminin appeared as short radial fibrils between the cells and the substratum, and in the pericellular matrix. Synthesis of fibronectin and laminin by 7-day keratinocyte cultures was determined by 18 hr ^{35}S-methionine metabolic labeling followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography. Fibronectin accounted for 2.9% of total synthesized protein, 26.5% of fluid phase protein secretion, and 4.3% of deposited ECM protein. In contrast, only 0.1% of the total synthesized protein was laminin, little (6.3%) of this product was secreted, and none of this product was deposited in the ECM. Our results indicate that human keratinocytes under culture conditions that prevent terminal differentiation in vitro can synthesize, secrete, and deposit fibronectin in the extracellular matrix. Although these cells synthesize laminin, they secrete very little and deposit no detectable laminin in the matrix under these culture conditions. From these data we believe that fibronectin may play an important role in the interaction of epidermal cells with connective tissue matrix during wound healing or morphogenesis in in vivo situations in which the epidermis is not terminally differentiated.

Key words: keratinocytes, fibronectin, laminin, extracellular matrix

During the re-epithelialization of an excisional skin wound, the epidermis migrates over a provisional matrix of fibronectin and fibrin [1] while the usual basement membrane and its constituents, laminin and type IV collagen, are absent from the dermal-epidermal junction. When human keratinocytes are grown in tissue culture under low Ca^{2+} conditions (0.1 mM or less), they express a phenotype that is similar to the migrating epidermal cells of a re-epithelializing wound. These similarities include absence of terminal differentiation [2,3], retracted tonofilaments [2,4], paucity of desmosomes [2,5], and presence of peripheral actin bundles [2,4].

Received July 3, 1984; accepted January 24, 1985.

In this report we provide evidence that the profile of extracellular matrix proteins produced by keratinocytes under low Ca^{2+} conditions in vitro reflect the provisional matrix observed under the migrating epithelium in vivo.

METHODS

Keratinocyte Cultures

Keratinocytes were isolated from human neonatal foreskins and cultured in serum-free, low-calcium medium (MCDB 153) [2].

Metabolic Labeling of Keratinocyte Cultures

Second- or third-passage keratinocytes were grown for 7 days in 35-mm tissue culture dishes (Costar, Cambridge, MA), then labeled overnight with 0.8 ml of methionine-free MCDB 153 plus 26 μCi ^{35}S-methionine (New England Nuclear, Boston, MA) per dish. Labeled media were collected in the presence of 1 mM phenylmethylsulfonylfluoride (PMSF, Calbiochem-Behring Co., La Jolla, CA) and centrifuged at 12,500 rpm for 20 min on a Brinkman Centrifuge 3200 to remove insoluble aggregates.

Monolayer Extract Procedure

Washed monolayers were removed from the tissue culture dishes with a rubber policeman in the presence of 1.0% Nonidet P40 (NP40) (Sigma, St. Louis, MO) and 5 mM EDTA in phosphate-buffered saline, pH 7.4 (PBS) plus an enzyme inhibitor cocktail that included 1 mM PMSF, 1 mM parachloromercuribenzoic acid, 1 mM benzamidine, and 1 mM EGTA. Insoluble particulates were removed by centrifugation at 12,500 for 20 min.

Cell and ECM Extract Procedure

Since the monolayer extract contains both extracellular matrix (ECM) proteins and intracellular proteins, an attempt was made to separate these two compartments by a modification [6] of the extraction technique of Hedman et al [7]. In brief, the cells were extracted with 0.5% desoxycholate (DOC, Fisher Scientific Co., Fairlawn, NJ) in Hanks' balance salt solution (HBSS) without Ca^{2+} and Mg^{2+}, pH 7.4 and the enzyme inhibitor cocktail described above. The residual proteins in the tissue culture dish were extracted twice with 50 μg/ml DNAse type I (Sigma) in HBSS and the enzyme inhibitor cocktail at 25°C for 15 min; then the ECM was removed with a rubber policeman in the presence of 1% NP-40 as described above.

TCA Precipitates

Proteins from monolayer, cell, and ECM extracts and media were precipitated with 10% trichloroacetic acid (TCA) overnight at 4°C. Acetone-washed precipitates were measured for radioactivity on a Beckman scintillation counter. Total synthesized protein was calculated on a per dish basis. To some samples 1 mg/ml of carrier protein (bovine serum albumin, BSA) was added to insure a complete precipitation of proteins from extracts and media. However, we found that carrier protein was not necessary for complete precipitation if the samples were not extensively diluted during the precipitation procedure. Therefore, samples to be analyzed by SDS-PAGE were TCA-precipitated without carrier.

Antiserum Production, Isolation, and Specificity

Antisera to human fibronectin, prepared by the method of Furie and Rifkin [8], and to mouse laminin, prepared by the method of Timpl et al [9] (Bethesda Research Laboratory, Fredricksburg, MD), were raised in rabbits and sheep by a modification of the immunization schedule of Crowle [10]. Antibodies were isolated by DEAE ion exchange chromatography and NH_4SO_4 precipitation [11].

The specificity of the antifibronectin antibodies (anti-FN) was demonstrated by the Western immunoblot technique [12] against human plasma (Fig. 1). Only fibronectin appears in the immunoblot of whole plasma (Fig. 1, tracks 2 and 3), and no band appears in the immunoblot of fibronectin-depleted plasma (Fig. 1, track 4). These data provide strong evidence that our rabbit anti-FN binds only fibronectin. In addition, fibronectin (and protein A) appears in the immunoblots of Staph protein A precipitates from cell extract and conditioned medium (Fig. 2, panel B, tracks 2 and 4), and no band appears in the immunoblots of TCA precipitates from fibronectin-depleted cell extract and conditioned medium (Fig. 2, panel B, tracks 3 and 5). These data provide strong evidence that our rabbit anti-human fibronectin has immunologic specificity only for fibronectin.

Keratinocyte laminin occurred in such small amounts that immunoblots were not possible; however, only bands at 440 kD and at 220 kD that comigrated with cold-carrier laminin were observed on antilaminin (anti-LM) immunoprecipitates of conditioned medium (Figure 3, tracks 4 and 5). In addition, the anti-LM antibodies gave a staining pattern on human skin sections that was typical of basement membrane and a strong reaction to laminin but no reaction to fibronectin or purified type IV collagen (Bethesda Research Laboratories) on enzyme-linked immunosorbant assay (ELISA) [13]. These data provide strong evidence that the anti-LM antibodies are immunologically specific for laminin.

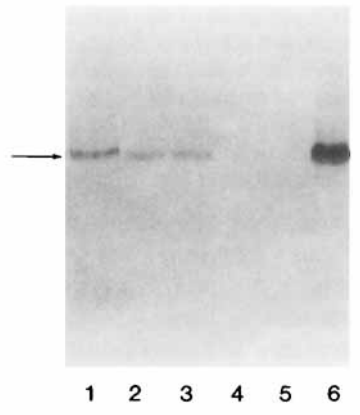

Fig. 1. Specificity of antifibronectin antibodies. Purified plasma fibronectin (tracks 1 and 6), whole human plasma (tracks 2 and 3), and fibronectin-depleted human plasma (track 4) after reduction were processed by 5% SDS-PAGE, electrophorectically transferred from SDS gel to nitrocellulose paper, and stained with rabbit anti-human fibronectin antibodies and ^{125}I-labeled goat anti-rabbit IgG second antibody. Tracks 1 and 6 represent two different preparations of plasma fibronectin after elution from a gelatin-Sepharose 4B affinity column. Track 4 represents a sample of plasma depleted of fibronectin by passage over a gelatin-Sepharose 4B affinity column. Track 5 contained molecular weight standards.

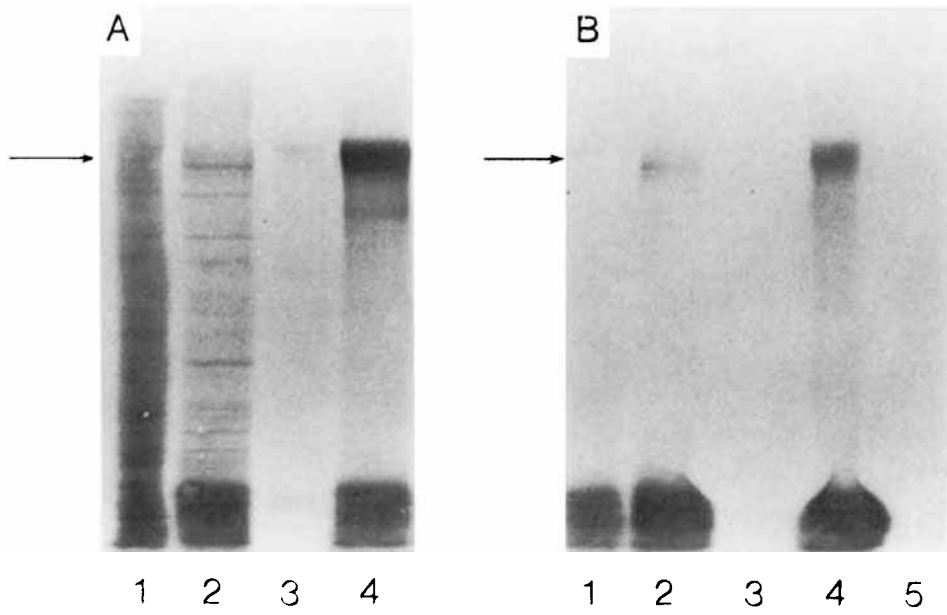

Fig. 2. A) A 5% reduced SDS gel autoradiograph of ^{35}S-methionine-labeled cell extracts (track 1), material bound to Staph protein A from cell extracts (track 2), conditioned media (track 3), and material bound to Staph protein A from conditioned medium (track 4). Staph protein A binds fibronectin (arrow) as well as other proteins from the cell extract (track 2) and conditioned medium (track 4). B) Immunoblot of a 5% reduced SDS gel containing Staph A precipitates and supernatants of keratinocyte extracts and conditioned media. Proteins were electrophorectically transferred from SDS gel to nitrocellulose paper and stained with rabbit anti-human fibronectin antibodies and ^{125}I-labeled goat anti-rabbit IgG second antibody. Track 1, Staph A run alone. Track 2, material bound to Staph A from cell extracts. Track 3, the TCA precipitate of the residual cell extract. Track 4, material bound to Staph protein A from conditioned media. Track 5, the TCA precipitate of the residual conditioned media. Protein A eluted off of Staphylococci, ran at the dye front, and bound the ^{125}I-labeled second antibody presumably at its Fc receptor, not its antibody binding site (track 1, also seen in tracks 2 and 4). All of the fibronectin (arrow) contained in cell extracts and conditioned media is removed by Staph A (tracks 2–5).

Immunoprecipitation of Conditioned Media and Extracts

Antigen-antibody immunoprecipitation curves were generated by the quantitation [14] of precipitated protein when increasing amounts of fibronectin or laminin were added to a fixed quantity of specific rabbit or sheep IgG antibodies. For immunoprecipitation of fibronectin or laminin from conditioned media and extracts, aliquots of samples were incubated with antibodies and cold antigen at equivalency (the level of cold antigen that gave maximal protein precipitation with a given quantity of antibodies). The immunoprecipitates were centrifuged through a discontinuous sucrose gradient (0.25 ml of 0.5 M sucrose and 0.5 ml of 1 M sucrose) in the presence of 1% Triton X-100, 1% SDS, and 1% DOC followed by four washes with 1% Triton X-100, 1% DOC, and 150 mM KCl to remove nonspecifically trapped material [15]. For documentation of radioactivity, immunoprecipitates were dissolved in 0.2 ml formic acid and 10 ml Ready-Solv (Beckman, Fullerton, CA) and quantitated in a Beckman liquid scintillation counter.

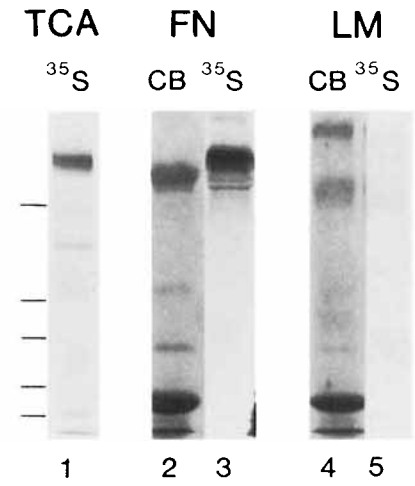

Fig. 3. SDS gel and corresponding autoradiograph composite of TCA precipitate of keratinocyte medium (track 1), immunoprecipitate of fibronectin (tracks 2 and 3), and immunoprecipitate of laminin (tracks 4 and 5). ^{35}S indicates autoradiograph of 5% reduced SDS gel and CB indicates Coomassie blue stain of the same SDS gel. Hatch marks indicate molecular weight standards of 205 kD, 116 kD, 97 kD, 66 kD, and 45 kD.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Autoradiography of Precipitates

TCA precipitates or immunoprecipitates were dissolved in sample buffer (62 mM Tris, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 0.001% bromophenol blue), boiled for 10 min, and examined by 5% SDS-PAGE containing a 7% acrylamide cushion on the bottom third of the gel [16]. Gels were electrophoresed overnight at 5 mA, stained with 0.1% Coomassie brilliant blue, and dried onto filter paper (Bio-Rad Laboratories, Richmond, CA) under vacuum. Autoradiography was performed by exposing Kodak X-omat AR film (Rochester, NY) to the dried SDS gel.

Densitometry Scans of SDS Gel Autoradiography

Since immunoprecipitates of monolayer extracts, cell extracts, and ECM extracts contained a large amount of nonspecifically trapped protein despite centrifugation through sucrose gradients and multiple Triton X-100 washes (see Methods above), densitometry scans were performed on the SDS gel autoradiographs (Figures 4–7). Each track was scanned with a densitometer (Ortec, Model 4310, Oak Ridge, TN). The tracings were photocopied, cut out, and weighed on a Mettler AC100 (Mettler Instruments Corp, Hightstown, NJ). The proportion of fibronectin or laminin in an immunoprecipitate was determined by dividing the weight of the specific protein peak(s) by the weight of the entire scan. Thus, the amount of radiolabeled fibronectin or laminin in an immunoprecipitate could be determined by multiplying the fraction present by the total radioactivity of the immunoprecipitate.

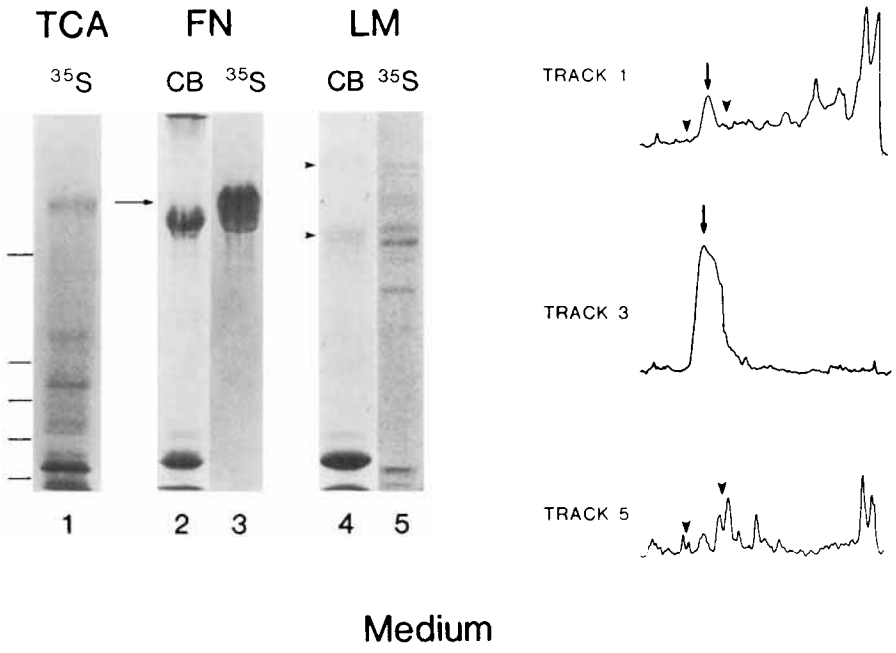

Fig. 4. SDS gel, autoradiograph, and autoradiograph densitometry scan composite of TCA precipitate of keratinocyte medium (track 1), immunoprecipitate of fibronectin (tracks 2 and 3), and immunoprecipitate of laminin (tracks 4 and 5). ^{35}S indicates autoradiograph of 5% reduced SDS gel and CB indicates Coomassie blue stain of the same SDS gel. Hatch marks indicate molecular weight standards of 205 kD, 116 kD, 97 kD, 66 kD, and 45 kD. Arrow at gel track 2 indicates molecular weight of keratinocyte fibronectin, which is larger than plasma fibronectin (shown in track 2). Arrowheads at track 4 indicate laminin heavy and light chain, which are the same size whether from keratinocyte cultures (track 5) or from EHS mouse sarcoma (track 4). Arrows in densitometry scans of tracks 1 and 3 indicate tracing peaks taken from fibronectin. Arrowheads in scans of tracks 1 and 5 indicate peaks taken for laminin. Autoradiographs photographed and scanned in tracks 1, 3, and 5 were exposed for 8 hr, 8 hr, and 6 days, respectively.

Avidin-Biotin Immunofluorescence Techniques on Keratinocyte Cultures

Third-passage keratinocytes were grown for 7 days in Lab-Tek culture chambers (Miles, Naperville, IL), then fixed with 2% paraformaldehyde and 1% NP40 if permeabilization of the cells was desired. After blocking nonspecific absorption with 20 mg/ml human serum albumin, the first antibody (sheep anti-FN at 18 μg/ml or sheep anti-LM at 50 μg/ml) was incubated with cultures for 1 hr at 37°C. Biotinylated second antibody (rabbit anti-sheep IgG at 5 μg/ml) was then incubated with the cultures for 1 hr at 37°C followed by 20 μg/ml FITC-avidin. Slides were mounted in 1:1 glycerol:PBS with 0.1% paraphenylenediamine. The latter markedly reduced quenching and counterstained the cells a light green [17].

RESULTS

To determine fibronectin and laminin synthesis by nonterminally differentiating human keratinocytes, we grew second- or third-passage cells for 7 days (approxi-

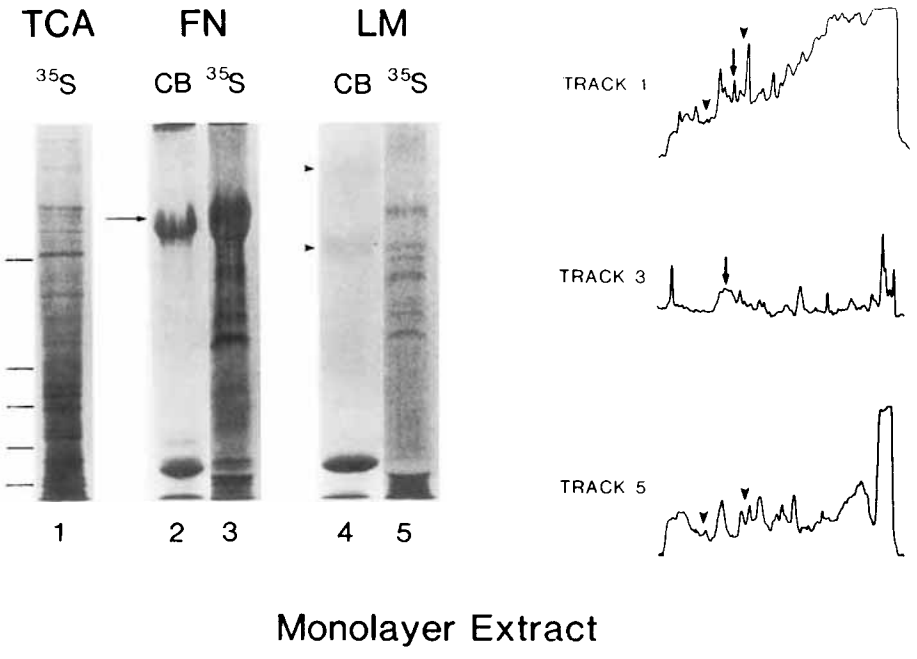

Fig. 5. SDS gel, autoradiograph, and autoradiograph densitometry scan composite of TCA precipitate of monolayer extract (track 1), immunoprecipitate of fibronectin (tracks 2 and 3), and immunoprecipitate of laminin (tracks 4 and 5). ^{35}S and CB denote autoradiograph and Coomassie blue stain of the same 5% reduced SDS gel, respectively. Hatch marks indicate molecular weight standards of 205 kD, 116 kD, 97 kD, 66 kD, and 45 kD. Arrow at gel track 2 indicates keratinocyte fibronectin and those in densitometry scans of tracks 1 and 3 indicate tracing peaks taken for fibronectin. Arrowheads at gel track 4 indicate laminin heavy and light chains and in densitometry scans of tracks 1 and 5 indicate tracing peaks taken for laminin. Autoradiographs photographed in tracks 1, 3, and 5 were exposed for 8 hr, 6 days, and 6 days, respectively, and autoradiographs scanned as shown in tracks 1, 3, and 5 were exposed for 8 hr, 20 days, and 6 days, respectively.

mately 80% confluence) in MCDB 153 and then metabolically labeled the cells with ^{35}S-methionine for 16–18 hr. Aliquots of both media and culture extracts were either TCA-precipitated or immunoprecipitated with the appropriate antibody and cold antigen carrier. However, measurement of fibronectin and laminin synthesis as a fraction of total protein synthesis (immunoprecipitate radioactivity/TCA precipitate radioactivity) was made difficult by entrapment of other cell proteins (Figs. 4–7, tracks 3 and 5) in the immunoprecipitates of culture extracts. This entrapment occurred despite the centrifugation of precipitates through a sucrose density gradient and washing precipitates with detergent-containing buffers (see Methods). Nevertheless, we felt the protein entrapment was nonspecific, since it appeared similarly in both fibronectin and laminin immunoprecipitates from culture extracts and since rigorous examination of antibody specificity was carried out (see Methods, Figs. 1–3).

To resolve this problem we initially fractionated culture extracts over Sephadex G150 columns and took the void volume peak for immunoprecipitation reactions with anti-FN and anti-LM. This procedure was successful since fibronectin and laminin,

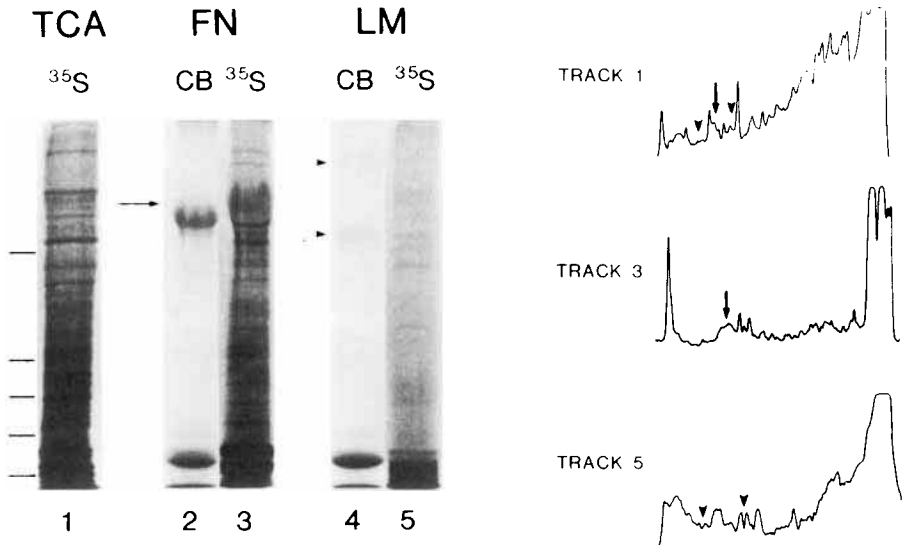

Fig. 6. SDS gel, autoradiograph, and autoradiograph densitometry scan of composite of TCA precipitate of soluble DOC extract of keratinocyte cell cultures (track 1), immunoprecipitate of fibronectin (tracks 2 and 3), and immunoprecipitate of laminin (tracks 4 and 5). ^{35}S and CB denote autoradiograph and Coomassie blue stain of the same 5% reduced SDS gel, respectively. Hatch marks indicate molecular weight standards of 205 kD, 116 kD, 97 kD, 66 kD, and 45 kD. Arrow at gel track 2 indicates keratinocyte fibronectin and those in densitometry scans of tracks 1 and 3 indicate tracing peaks taken for fibronectin. Arrowheads at gel track 4 indicate laminin heavy and light chains and in densitometry scans of tracks 1 and 5 indicate tracing peaks taken for laminin. Autoradiograph photographed in tracks 1, 3, and 5 were exposed for 20 hr, 6 days, and 6 days, respectively, and autoradiographs scanned as shown in tracks 1, 3, and 5 were exposed for 8 hr, 20 hr, and 6 days, respectively.

which are of approximately 0.5 and 1.0 million daltons, respectively, appeared in the void volume, while most cytoskeletal protein, which are 40–70 kilodaltons (kD), appeared at about one-half bed volume. Nevertheless, we found this methodology extremely cumbersome and time-consuming when we processed multiple extracts; therefore, we adopted a procedure in which the SDS gel autoradiographs of all immunoprecipitates are scanned by a densitometer to give the fraction of isotopic label in the immunoprecipitate that was attributable to either fibronectin or laminin (Table I and Table II, Figures 4–7). The two methodologies gave similar results (data not shown).

Culture extracts for these determinations were collected by two different protocols. In the first, medium was removed from the cultures and the cell monolayer (cell + ECM) was extracted in 1% NP40 (see Methods). In the second approach, the medium was removed, the cells were extracted with DOC, and then the ECM was extracted with 1% NP40 [7] (see Methods). When these two extraction techniques were used in parallel, essentially the same results were obtained in terms of total fibronectin and laminin synthesis (compare Table I and Table II). The media were pooled from the cell cultures that were used in the two extraction procedures.

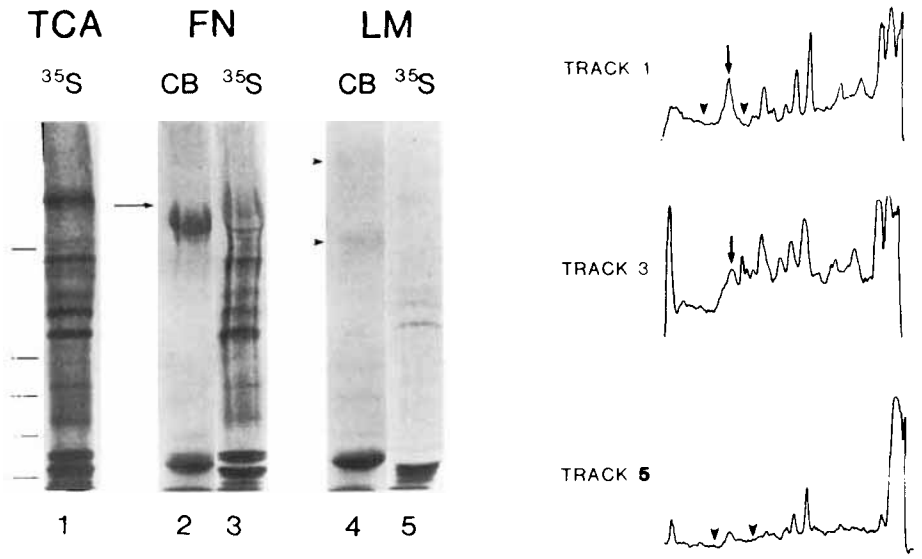

Fig. 7. SDS gel, autoradiograph, and autoradiograph densitometry scan composite of TCA precipitate of ECM extract (track 1), immunoprecipitate of fibronectin (tracks 2 and 3), and immunoprecipitate of laminin (tracks 4 and 5). ^{35}S and CB denote autoradiograph and Coomassie blue stain of the same 5% reduced SDS gel, respectively. Hatch marks indicate molecular weight standards of 205 kD, 116 kD, 97 kD, 66 kD and 45 kD. Arrow at gel track 2 indicates keratinocyte fibronectin and those in densitometry scans of tracks 1 and 3 indicate tracing peaks taken for fibronectin. Arrowheads at gel track 4 indicate laminin heavy and light chains and in densitometry scans of tracks 1 and 5 indicate tracing peaks taken for laminin. Autoradiographs photographed in tracks 1, 3, and 5 were all exposed for 20 hr, and autoradiographs scanned as shown in tracks 1, 3, and 5 were exposed for 8, 20, and 20 hr, respectively.

To derive fibronectin and laminin synthesis as a percentage of total protein synthesis using the first extraction protocol, the sum of medium and monolayer radioactivity attributable to either fibronectin or laminin was divided by the sum of TCA radioactivity (total protein) and multiplied times 100 (Table I). Figures 4 and 5 show the SDS gel autoradiographs and corresponding densitometry scans that were used to derive these data. Likewise, to derive fibronectin and laminin synthesis as a percentage of total protein synthesis by the second extraction protocol, the sum of medium, DOC-soluble cell extract, DOC-lifted particulates, and NP40 ECM extract radioactivity attributable to either fibronectin or laminin was divided by the sum of TCA radioactivity and multiplied times 100 (Table II). Figures 4, 6, and 7 show the SDS gel autoradiographs and corresponding densitometry scans that were used to derive these data.

Thus, when 7-day keratinocyte cultures at approximately 80% confluence were metabolically labeled with ^{35}S-methionine for 18 hr and extracted by the two procedures described above, the synthesis of fibronectin accounted for 2.9% (Table I) or 3.2% (Table II) of the total protein synthesis, whereas that of laminin accounted for only 0.1% or 0.4%. The amounts of newly synthesized fibronectin and laminin that were secreted into fluid phase (medium) can also be derived from these data. The

TABLE I. 18-hr Protein Synthesis by Keratinocyte Cultures Derived From Monolayer Extracts

	TCA precipitate	Immunoprecipitate	
		FN	LM
Medium			
Total cpm[a]	0.94×10^6	300,000	9,650
X Scan fraction[b]		0.83	0.23
cpm		249,000	2,220
Monolayer			
Total cpm	9.35×10^6	240,000	122,800
X Scan fraction		0.21	0.077
cpm		50,400	9,500
Totals[c]	10.29×10^6	299,400	11,720
%Total protein[d]	100%	2.9%	0.1%

[a]Total cpm per dish.
[b]Densitometry scan fractions of fibronectin and laminin in an immunoprecipitate were determined by dividing the weight of the specific protein peak(s) by the weight of the entire scan (see Materials and Methods).
[c]Sum of medium and monolayer cpm.
[d]Quotient of total FN or LM cpm divided by total TCA cpm × 100.

TABLE II. 18-hr Protein Synthesis by Keratinocyte Cultures Derived From Cell and ECM Extracts

	TCA precipitate	Immunoprecipitate	
		FN	LM
Medium (see Table I)	0.94×10^6	249,000	2,220
DOC-soluble cell extract			
Total cpm[a]	8.43×10^6	1,170,000	693,300
X Scan fraction[b]		0.053	0.044
cpm		62,000	30,500
DOC-lifted participates[c]			
Total cpm	0.41×10^6	100,000	122,500
X Scan fraction		0.010	0.021
cpm		1,000	2,570
NP40 ECM extract			
Total cpm	0.22×10^6	140,000	69,660
X Scan fraction		0.068	0
cpm		9,520	0
Totals[d]	10.0×10^6	321,520	35,290
%Total protein[e]	100%	3.2%	0.4%

[a]Total cpm per dish.
[b]Densitometry scan fractions of fibronectin and laminin in an immunoprecipitate were determined by dividing the weight of the specific protein peak(s) by the weight of the entire scan (see Materials and Methods).
[c]DOC-lifted particulates were solubilized with 1% NP40 prior to precipitation procedures.
[d]Sum of medium, DOC-soluble cell extract, DOC-lifted particulates, and NP40 ECM extract.
[e]Quotient of total FN or LM cpm divided by total TCA cpm × 100.

radioactivity in the medium attributable to fibronectin or laminin divided by the TCA-precipitable radioactivity in the medium (see line 1 of Table I or II) gives the percentage of total protein secreted into the medium that was fibronectin and laminin. As shown in Table III, fibronectin accounted for 26.5% of the total protein secreted while laminin accounted for only 0.2% secreted. Preliminary results using ^{14}C mixed amino acids for metabolic labeling gave essentially the same results for fibronectin synthesis and secretion but about one-half the amount of laminin synthesis and secretion (data not shown).

The amounts of newly synthesized fibronectin and laminin that were deposited into the ECM can also be derived as outlined above. As shown in Table III fibronectin accounted for 4.3% of the total protein deposited in ECM, but laminin was undetectable. This result agrees with the presence of fibronectin and the absence of laminin in the pericellular matrix as demonstrated by the immunofluorescence technique (see Fig. 8, panels A and C). We believe, however, that the 4.3% calculation greatly underestimates the percentage of fibronectin in the ECM, since the SDS gel autoradiograph of the TCA precipitate of ECM extract shows many low-molecular-weight protein bands that are characteristic of cytoskeletal protein (Fig. 7, track 1).

Finally, the amounts of newly synthesized fibronectin and laminin that were retained in the cells can be derived. The sum of the radioactivity in the DOC-soluble cell extract and the DOC-lifted particulates attributable to fibronectin or laminin divided by the sum of TCA-precipitable radioactivity in the DOC-soluble cell extract and the DOC-lifted particulates (see Table II, lines 2 and 3) gives the percentage total protein retained in the cells that was fibronectin and laminin. As shown in Table III, fibronectin and laminin accounted for 0.7% and 0.3% of the total newly synthesized protein retained in the cells. This result corresponds to the presence of both fibronectin and laminin in intracellular granules as demonstrated by immunofluorescence of permeabilized keratinocyte cultures (see Fig. 8, panels B and D). Thus, under these culture conditions most newly synthesized fibronectin was either secreted into the fluid phase or deposited in the ECM, but most newly synthesized laminin was retained by the cells.

The percentage of fibronectin and laminin in the various compartments of the DOC extraction procedure can be derived from the data shown in Table II by dividing

Table III. Total Synthesis, Fluid Phase Secretion, and ECM Deposition of Fibronectin and Laminin by Human Keratinocytes

	Synthesis[a] (% of total protein)	Secretion[b] (% of protein secreted into medium)	Deposition[c] (% of protein deposited in ECM)	Retained[d] (% of protein retained in cells)
Fibronectin	2.9 (3.2)	26.5	4.3	0.7
Laminin	0.1 (0.4)	0.2	Undetectable	0.3

[a]See Table I. Data in parentheses were derived from Table II.
[b]See Table I or II. Quotient of FN or LM cpm in medium divided by TCA cpm in medium × 100, respectively.
[c]See Table II. Quotient of FN or LM cpm in ECM divided by TCA cpm in ECM, × 100, respectively.
[d]See Table II. Quotient of FN or LM cpm in DOC soluble cell extract plus DOC-lifted particulates divided by TCA cpm in DOC-soluble cell extract plus DOC-lifted particulates × 100, respectively.

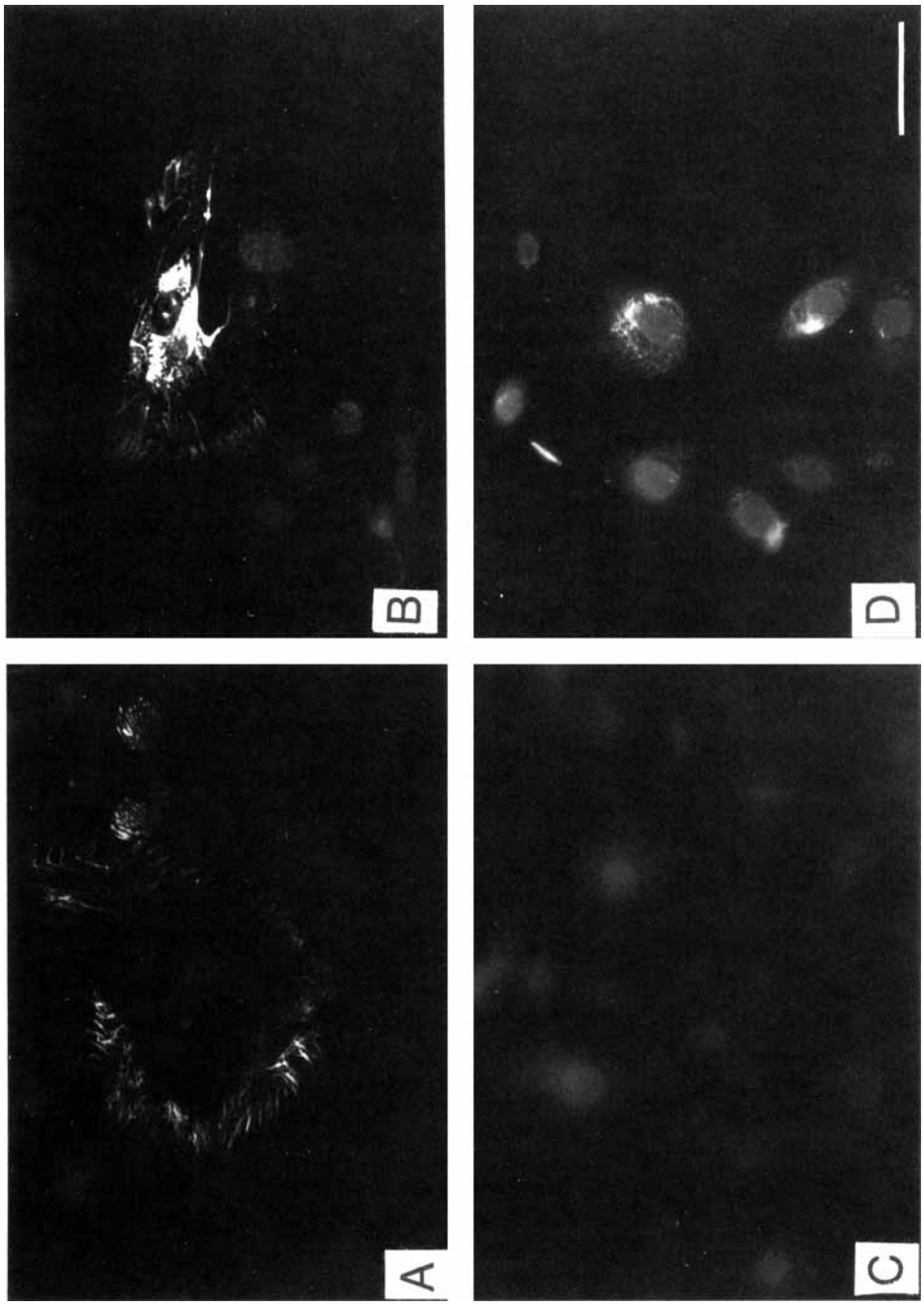

Fig. 8.

the radioactivity in a particular compartment by the total radioactivity attributable to fibronectin or laminin. As shown in Table IV, 19.6% of newly synthesized fibronectin was retained in the cells, while 77.4% was secreted into the medium and 3.0% was deposited in the ECM. In contrast, 93.7% of newly synthesized laminin was retained in the cells, whereas only 6.3% was secreted into the medium and no detectable amount was deposited in the ECM.

SDS-PAGE Autoradiography of Fibronectin and Laminin

Autoradiographs of the TCA precipitates and immunoprecipitates from keratinocyte-conditioned medium and extracts are shown in tracks 1, 3, and 5 of Figures 3–7. In the TCA precipitate of conditioned medium (Figs. 3 and 4, track 1), a major protein band was seen at 240–250 kD that could be immunoprecipitated with antifibronectin antibodies (Figs. 3 and 4, track 3). Thus, the major protein band in conditioned medium appears to be fibronectin. A very small amount of laminin can also be immunoprecipitated from conditioned medium (Figs. 3 and 4, track 5). The autoradiographic data in Figure 3 correlated well with the fibronectin and laminin secretion data based on precipitable radioactivity (data not shown). However, the autoradiographic data in track 5 of Figure 4 demonstrated that direct quantitation of laminin secretion from immunoprecipitable radioactivity would overestimate the amount of laminin because of contamination with other proteins. Therefore, densitometry scans of the SDS-gel autoradiographs were performed (Figs. 4–7) as previously discussed.

In the TCA precipitate of cell monolayers (Fig. 5, track 1) a minor doublet was seen at 240–250 kD that was totally immunoprecipitable with antifibronectin (Fig. 4,

TABLE IV. Compartmentalization of Newly Synthesized Fibronectin and Laminin in Keratinocyte Cultures

	Medium[a] (%)	ECM[b] (%)	Cells[c] (%)
Fibronectin	77.4	3.0	19.6
Laminin	6.3	0	93.7

[a]See Table II. Quotient of FN or LM cpm in medium divided by total FN or LM cpm, respectively, × 100.
[b]See Table II. Quotient of FN or LM cpm in ECM extract divided by total FN or LM cpm, respectively, × 100.
[c]See Table II. Quotient of FN or LM cpm in DOC soluble cell extract plus DOC lifted particulates divided by total FN or LM cpm, respectively × 100.

Fig. 8. Immunofluorescence photomicrograph of keratinocyte culture that had been fixed (A, C) or fixed and permeabilized (B, D) and stained with A'FN (A, B) or A'LM (C, D) by the FITC-avidin-biotin technique (see Materials and Methods). A) Fixed, nonpermeabilized keratinocytes have fine radial fibrils of fibronectin in a pericellular distribution. B) Fixed, permeabilized keratinocytes demonstrate both the extracellular fibrils and brightly staining intracellular granules of fibronectin. C) Fixed, nonpermeabilized keratinocytes have no observable laminin in the extracellular matrix. D) Fixed, permeabilized keratinocytes show moderately staining intracellular granules. Bar = 10 μm.

track 3). Laminin was barely discernible in the TCA precipitate of the monolayer; however, it could be readily detected at 440 kD and 220 kD after immunoprecipitation with antilaminin antibodies (Fig. 4, track 5). Similar results were obtained with SDS autoradiography of DOC cell extracts (Fig. 5).

When the banding pattern of the TCA precipitate of cell monolayer extract (Fig. 5, track 1) is compared to the TCA precipitate banding pattern of DOC cell extract (Fig. 6, track 1), they are seen to be almost identical. This indicates that the majority of bands in the cell monolayer are attributable to intracellular proteins not ECM. In fact, intracellular protein radioactivity is approximately 40 times the radioactivity of ECM (Table II). In contrast, when the banding pattern of the ECM TCA precipitate (Fig. 7, track 1) is compared to either former pattern (track 1, Figs. 5 and 6), a marked difference is noted. Fibronectin was immunoprecipitated from the ECM extract (Fig. 7, track 3), but no appreciable laminin was immunoprecipitated. This finding is presented in tabular form in Tables III and IV.

Immunofluorescence of Keratinocyte Fibronectin and Laminin

To check our biochemical data, we stained intact and permeabilized keratinocyte cultures for fibronectin and laminin to examine morphologically the intracellular and extracellular distribution of those two proteins. As demonstrated in panels A and B of Figure 8, fibronectin appears extracellularly as radial fibrils perpendicular to the perimeter of cells, and intracellularly as perinuclear granules. In contrast, laminin is not present in the extracellular matrix but does occur intracellularly in perinuclear granules at almost half of the intensity of granules stained for fibronectin (Fig. 8, panels C and D). These results correlate completely with the biochemical summary presented in Tables III and IV.

DISCUSSION

During the re-epithelialization of an excisional skin wound, the epidermis migrates over a provisional matrix of fibronectin and fibrin [1], whereas the usual basement membrane [3] and its constituents, laminin and Type IV collagen, are absent from the dermal-epidermal junction [1,18]. In part, the fibronectin in this provisional matrix comes from in situ synthesis [19]. Whether this fibronectin comes from the migrating, nonterminally differentiating epithelium above or from underlying fibroblasts is unknown; however, human keratinocytes cultured in low Ca^{2+} conditions have been shown to be nonterminally differentiating [2] and to synthesize, secrete, and deposit fibronectin in the pericellular matrix [20,21]. In contrast, while human keratinocytes have been demonstrated to synthesize and secrete laminin [22], Kariniemic et al [23] could not demonstrate laminin in the extracellular matrix.

In this report we have quantitatively demonstrated by biochemical techniques (Tables I–IV, Figs. 4–7) that under low Ca^{2+} culture conditions human keratinocytes a) synthesize both fibronectin and laminin, b) secrete fibronectin in large quantities and laminin in small quantities into the media, and c) deposit fibronectin but not laminin in the pericellular matrix. The deposition of fibronectin but not laminin in the pericellular matrix was confirmed morphologically by FITC-avidin-biotin immunofluorescence techniques (Fig. 8).

We surmise that the in vitro pericellular deposition of fibronectin, but not laminin, by keratinocytes cultured in low Ca^{2+} may reflect the in vivo presence of

fibronectin, but not laminin, in the provisional matrix under the migrating epithelium during wound repair.

ACKNOWLEDGMENTS

We thank Jonathan Baskin and Richard Ham for providing human keratinocytes for these studies, Marialyce Austin for her excellent typing assistance, and Barry Silverstein for his expert photo illustration assistance.

This work was supported by National Institutes of Health grant Am 31514.

REFERENCES

1. Clark RAF, Lanigan JM, Dellapella P, Manseau E, Dvorak HF, Colvin RB: J Invest Dermatol 79:264–269, 1982.
2. Boyce ST, Ham RG: J Invest Dermatol 81 (Suppl):33s–40s, 1983.
3. Odland G, Ross R: J Cell Biol 39:135–151, 1967.
4. Gabbiani G, Chaponnier C, Huttner I: J Cell Biol 76:561–568, 1978.
5. Krawczyk WS, Wilgram GF: J Ultrastruct Res 45:93–101, 1973.
6. Hormia M, Lehto V-P, Virtanen I: Exp Cell Res 149:483–497, 1983.
7. Hedman K, Kurkinen M, Alitalo K, Vaheri A, Johansson S, Hook J: Cell Biol 81:83–91, 1979.
8. Furie MB, Rifkin DB: J Biol Chem 255:3134–3140, 1980.
9. Timpl R, Rohde H, Robey PG, Rennard SI, Foidart J-M, Martin GR: J Biol Chem 254:9933–9937, 1979.
10. Crowle AJ: "Immunodiffusion." New York: Academic, 1973, pp 85–88.
11. Williams CA, Chase MW: "Methods of Immunology and Immunochemistry." New York: Academic Press, Vol 1, 1967, p 422.
12. Towbin H, Staehelin T, Gordon J: Proc Natl Acad Sci USA 76:4350–4354, 1979.
13. Engvall E, Perlmann P: Immunochemistry 8:871–874, 1971.
14. Lowery OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265–275, 1951.
15. Rhoads RE, McKnight GS, Schimke RT: J Biol Chem 248:2031–2039, 1973.
16. Laemmli UK: Nature 227:680–685, 1970.
17. Huff JC, Weston WL, Wanda KD: J Invest Dermatol 78:449–450, 1982.
18. Stanley JR, Alvarez OM, Berk EW Jr, Eaglestein WH, Katz SI: J Invest Dermatol 74:54–58, 1980.
19. Clark RAF, Winn HJ, Dvorak HF, Colvin RB: J Invest Dermatol 80 (Suppl):26s–30s, 1983.
20. Kubo M, Norris DA, Howell SE, Ryan SR, Clark RAF: J Invest Dermatol 82:580–586, 1984.
21. O'Keefe EJ, Woodley D, Castillo G, Russell N, Payne RE: J Invest Dermatol 82:150–155, 1984.
22. Alitalo K, Kuismanen E, Myllyla R, Khstala U, Asko-Seljavaara S, Vaheri A: J Cell Biol 94:497–505, 1982.
23. Kariniemi A-L, Lehto V-P, Vartio T, Virtanen I: J Cell Sci 58:49–61, 1982.