0022-202X/84/8202-0150$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 82:150–155, 1984

Vol. 82, No. 2
Printed in U.S.A.

Production of Soluble and Cell-Associated Fibronectin by Cultured Keratinocytes

EDWARD J. O'KEEFE, M.D., DAVID WOODLEY, M.D., GABRIELLE CASTILLO, M.D., NANCY RUSSELL, B.S., AND RICHARD E. PAYNE, JR., B.S.

Department of Dermatology, University of North Carolina School of Medicine, Chapel Hill, North Carolina, U.S.A.

Fibronectin has been demonstrated in epithelial cell types in culture, but published studies of keratinocytes have shown patterns of fibronectin produced by cells grown in medium with serum, which contains fibronectin. Since plasma fibronectin can bind to cells in vitro, cells grown in serum-supplemented media could show artifactual patterns of cell-associated fibronectin. To study insoluble fibronectin produced by keratinocytes, we plated cells in the absence of feeder layers in medium lacking fibronectin. Medium conditioned by metabolically labeled keratinocytes was studied by immunoprecipitation and by extraction with gelatin-Sepharose. Cells grown in fibronectin-free medium were labeled using affinity-purified anti-fibronectin antibody and fluorescein-conjugated antirabbit IgG. Keratinocytes produced soluble fibronectin, since both immunoprecipitation and adsorption to gelatin-Sepharose detected ^{35}S-methionine-labeled material which comigrated with human plasma fibronectin on sodium dodecyl sulfate polyacrylamide gels. Demonstration of insoluble, cell-associated fibronectin was enhanced in Triton X-100-extracted cells and was seen in subcellular fibrillar arrays at both physiologic and reduced Ca^{++} concentrations, but in intracellular locations only at physiologic Ca^{++} concentrations. When cells grown in 1.1 mM Ca^{++} were removed with Triton X-100, diffusely distributed fibrillar fibronectin remained on the surface of the coverslip. Asymmetric "tracks" of fibronectin left by sparsely plated cells suggested movement. Fibronectin is deposited by keratinocytes on the culture surface and may be modulated by culture conditions.

Fibronectin, a 440,000 dalton glycoprotein, is a major component of the extracellular matrix produced by fibroblasts and is able to bind to cells and other components of the matrix (collagen, glycosaminoglycans, and probably proteoglycans [1–4]). Although the cells producing the largest amounts of fibronectin are fibroblasts, production of extracellular fibronectin beneath cultured cells has been described for some epithelial cell types (liver [5,6], gut [7], kidney [8], and mammary [9,10]). These studies were usually performed in serum-containing media; since soluble fibronectin can be demonstrated to bind to the matrix both in vivo [11] and in vitro [12–15], the possibility that fibronectin demonstrated by immunofluorescence around epithelial cells was absorbed from the medium cannot be excluded. Since immunofluorescence has not shown fibronectin to be present in epithelia in vivo [16], and since a careful study of the basement membrane zone has indicated that fibronectin is absent from this area in mature, nonhealing epithelium [17], the biologic role of fibronectin of epithelial origin is unknown.

The presence of fibronectin in keratinocytes is also not established clearly in the literature. An initial study [18] was performed on cells cultured with a fibroblast feeder layer after fibroblasts had been removed and was performed in serum-containing medium. Four major reviews do not describe fibronectin production by keratinocytes [1–4]. Recent studies by Kariniemi et al [19] and by Peehl and Stanbridge [20] show apparent keratinocyte fibronectin by immunofluorescence, but both were performed on cells grown in medium containing fibronectin. Kariniemi et al show synthesis of metabolically labeled fibronectin in keratinocytes cultured without fibroblasts, confirming the report of Alitalo et al [18] performed under less stringent conditions.

We have identified fibronectin production by keratinocytes subcultured in the absence of fibroblasts by immunofluorescence and by metabolic labeling. To prevent contribution of serum fibronectin to the insoluble matrix, we removed fibronectin from the culture medium.

METHODS AND MATERIALS

Cell Culture

Swiss 3T3 cells were cultured in Dulbecco-Vogt modified Eagle's medium (DME) with 5% fetal bovine serum (Gibco Laboratories, Grand Island, New York), 50 μg of streptomycin, and 50 units penicillin per ml. Epidermal keratinocytes were prepared and cultured on mitomycin-C treated Swiss 3T3 feeder layers until confluent, according to Rheinwald and Green [21] with modifications as described [22], and any remaining 3T3 cells were removed with EDTA-containing buffers. Keratinocytes were then subcultured without feeder layers as previously described [22] in medium composed of ½ Ham's F-12 and ½ DME with a final concentration of fetal bovine serum of 2.5%. Epidermal keratinocytes were also plated and grown at reduced Ca^{++} concentrations in Joklik's modification of Eagle's medium with 5% fetal bovine serum extracted batchwise with Chelex 100 (Bio-Rad, Rockville Centre, New York) according to Brennan et al [23]. For immunofluorescence studies keratinocytes were cultured on glass coverslips in medium depleted of fibronectin by passage twice over a gelatin-Sepharose column. No differences in plating efficiency or growth were noted in fibronectin-depleted serum.

Purification and Coupling of Proteins

Fibronectin was purified from human plasma according to Hayashi and Yamada [24]. For antibody production 1 mg of fibronectin eluted from a gelatin-Sepharose column was electrophoresed on a preparative sodium dodecyl sulfate (SDS) polyacrylamide slab gel and the fibronectin band identified on strips cut out from the ends and center of the slab and stained with Coomassie blue. The remaining band containing fibronectin was cut out and mixed with complete Freund's adjuvant, homogenized in a Brinkmann Polytron, and injected intracutaneously into the flank of a rabbit. The rabbit was boosted twice at monthly intervals with similar amounts of fibronectin-containing gel in incomplete Freund's adjuvant and bled, and antibody was purified on a fibronectin-Sepharose column by elution with 1 M acetic acid into

Manuscript received July 11, 1983; accepted for publication October 14, 1983.

This work was supported by grant AM 25876 from the National Institutes of Health. Dr. O'Keefe is the recipient of a Research Career Development Award (AM 00977) from the National Institutes of Health.

Reprint requests to: E. J. O'Keefe, M.D., Department of Dermatology, Room 137, North Carolina Memorial Hospital, Chapel Hill, North Carolina 27514.

Abbreviations:
DME: Dulbecco-Vogt modification of Eagle's medium
PBS: Dulbecco's phosphate-buffered saline
SDS: sodium dodecyl sulfate

tubes containing 1 M unbuffered Tris. The antibody was dialyzed against 10 mM sodium phosphate, pH 7.5, containing 150 mM NaCl, diluted to A_{280} = 1.1, stored at −20°C, and used for all studies. The antibody stained characteristic fibronectin arrays by immunofluorescence in cultured human and mouse fibroblasts and identified fibronectin, but no other serum proteins, on immunoblots (see below). Sepharose 4BCL was activated with cyanogen bromide and coupled to fibronectin or gelatin by the method of Parikh et al [25].

Immunofluorescence

Immunofluorescence studies were performed on cells grown on glass coverslips without feeder layers, fixed in 3% formaldehyde in Dulbecco's phosphate-buffered saline (PBS), and extracted for 30 min at 24°C with 1% Triton X-100 in PBS. Cells were incubated with affinity-purified anti-fibronectin antibody (diluted 1:100 in PBS), anti-keratin antibody (1:50), or normal rabbit IgG (1:100) for 30 min at 24°C, washed 3 times for 5 min in PBS, incubated in fluorescein- or rhodamine-conjugated goat antirabbit IgG (Cappel Laboratories, West Chester, Pennsylvania) for 30 min at 24°C, washed, and mounted in 50% glycerol. Preparations were viewed in a Leitz Orthoplan microscope fitted with epifluorescence optics and appropriate filters and photographed with Kodak Tri-X film developed in Diafine (Acufine, Inc., Chicago, Illinois).

Electrophoresis and Immunoblots

Samples for SDS gel electrophoresis were dissolved in sample buffer as described in the figure legends and electrophoresed according to Fairbanks et al [26] in 3–17% gradient gels (exponential gradient maker, Hoefer Scientific Instruments, San Francisco, California) under reducing conditions as described [27]. Proteins were transferred to nitrocellulose paper by the method of Towbin et al [28] except that the tank buffer contained 20 mM Tris, 10 mM sodium acetate, 1 mM EDTA, and 0.05% SDS adjusted to pH 7.4. The nitrocellulose sheets with transferred proteins were then incubated with affinity-purified anti-fibronectin antibody diluted 1:100 in 25 mM Tris, 150 mM NaCl, 0.1% Triton X-100, pH 7.5 (blot buffer) containing 3% bovine serum albumin for 2 h at 24°C, washed in blot buffer twice for 5 min and then in 2 M urea, 0.1 M glycine, 1.0% Triton X-100, pH 7.5, for 5 min. The transfer was rinsed in blot buffer and incubated with ^{125}I-labeled protein A (200,000 cpm per ml) in blot buffer with 3% albumin according to Burnette [29], then rinsed sequentially in blot buffer and 2 M urea for a total of 3 washes as above. The transfer was blotted dry and an autoradiogram made after an 8- to 24-h exposure of Kodak XAR-5 X-ray film at −70°C with Dupont Cronex Lightning-Plus intensifying screens. Protein A was iodinated by the method of Hunter and Greenwood [30] to a specific activity of 5×10^4 dpm per ng.

Metabolic Labeling Studies

To demonstrate the synthesis of fibronectin by keratinocytes, we subcultured confluent cultures of keratinocytes in 35-mm culture dishes without 3T3 feeder layers in medium composed of ½ Ham's F-12 and ½ DME with 2.5% fetal bovine serum. (DME with 5% fetal bovine serum was conditioned for 24 h by 3T3 cells and combined with an equal volume of Ham's F-12. Final fetal bovine serum concentration was 2.5%.) The medium was then changed to ½ Ham's F-12 and ½ Hanks' balanced salt solution with 2.5% fetal bovine serum, and 50 μCi of [^{35}S]methionine was added. After 24 h medium was collected and the labeled and unlabeled fibronectin extracted by adsorption to gelatin-Sepharose or by immunoprecipitation.

One hundred microliters of a 50% slurry of gelatin-Sepharose was incubated with 300 μl of supernatant for 16 h at 4°C. The beads were washed 4 times with blot-buffer and then heated at 100°C for 3 min in electrophoresis sample buffer. The supernatant from the beads (20 μl) was electrophoresed on an SDS gel and the gel stained, dried, and used to expose Kodak XAR-5 X-ray film at −70°C for 5 days.

Labeled fibronectin was immunoprecipitated with 10 μg anti-fibronectin antibody or 10 μg rabbit IgG (control) in 200 μl of medium conditioned for 24 h by keratinocytes. Medium was incubated with the reagents for 60 min at 37°C in buffered detergent solutions as described by Choi and Hynes [31]. Goat antirabbit IgG (Cappel Laboratories, West Chester, Pennsylvania) (100 μl) was added for 60 min at 37°C and then incubated for 16 h at 4°C after addition of 5 μl normal rabbit serum as carrier. The precipitates were washed 3 times by centrifugation at 4000 g for 20 min, dissolved in electrophoresis sample buffer, and electrophoresed and stained. Autoradiograms were prepared as above.

Materials

Rabbit anti-keratin antiserum was a gift of Dr. T.-T. Sun. SDS, acrylamide and other reagents for polyacrylamide gels were from Bio-Rad (Rockville, Centre, New York). Freund's adjuvant was from Gibco (Grand Island, New York). Sepharose 4BCL was from Pharmacia (Piscataway, New Jersey). [^{125}I]NaI was from Amersham (Arlington Heights, Illinois) and [^{35}S]methionine from New England Nuclear (Boston, Massachusetts). Chemicals were from Fisher Scientific (Pittsburgh, Pennsylvania). Culture media and plastic ware were from Flow Laboratories (McLean, Virginia).

RESULTS

In order to document that keratinocyte cultures were making fibronectin, we incubated cells for 24 h with [^{35}S]methionine and detected labeled fibronectin by adsorption of the labeled material secreted into the culture medium to gelatin-Sepharose or by immunoprecipitation. Polyacrylamide gels of the eluate from gelatin-Sepharose beads incubated with culture supernatants from metabolically labeled cells showed a 220,000 dalton band which comigrated with the Coomassie blue-stained doublet produced by fibronectin adsorbed from fetal bovine serum in the medium (Fig 1). Furthermore, immunoprecipitation of fibronectin by affinity-purified anti-fibronectin antibody could be demonstrated with these supernatants (Fig 1). Smaller amounts of labeled material were seen in immunoprecipitates of detergent-insoluble material, which may represent cell-associated fibronectin [15] (data not shown).

Although metabolic labeling studies by cultured keratinocytes showed the synthesis of fibronectin in vitro, it did not exclude the possibility that the fibronectin which might be visualized by immunofluorescence was not synthesized but was extracted from the medium by cells. We therefore depleted fetal bovine serum of fibronectin by passage twice over a gelatin-Sepharose column. The column was eluted between passages with 4 M urea in 0.1 M glycine. After the second passage over the column, subsequent elution with 4 M urea yielded no protein peak detectable by absorbance at 280 nm, suggesting that all fibronectin had been removed. Electrophoretic blots [28] of serum before and after extraction twice by gelatin-Sepharose columns were developed with affinity-purified anti-fibronectin antibody followed by ^{125}I-labeled protein A [29]. Although Coomassie blue-stained gels of extracted and unextracted sera were identical, autoradiograms demonstrated that fibronectin had been removed from the extracted serum samples (Fig 2). Extracted serum showed no fibronectin band (Fig 2*E*), but unextracted serum showed a heavy band (Fig 2*F*) which comigrated with the fibronectin standard. Since fibronectin was synthesized by the cultures, and since the cultures shown were grown in fibronectin-free serum, we concluded that the material visualized by immunofluorescence was synthesized by the cells.

Extraction with detergent such as Triton X-100 was required for demonstration of keratinocyte fibronectin, possibly because of its location beneath the cells, but not for demonstration of fibroblast fibronectin. Identical results were obtained in cells treated with acetone or methanol at −20°C. Only small amounts of fibronectin were seen in unextracted cultures. Sparsely plated keratinocytes were associated with asymmetric or linear patterns of material staining for fibronectin deposited on the glass culture surface (Fig 3*A*,*B*). In some cases a keratinocyte could be visualized at the end of such a pattern, but often no cell was present in our preparations, since Triton X-100 often removed loosely adherent cells. These striking patterns gave the impression of movement and appeared to be "tracks" where a cell had moved from one point to another.

Extracellular fibronectin in cultures grown in 1.1 mM Ca^{++} also appeared to be deposited diffusely on the surface of the coverslips. In areas where cells were removed during treatment of the coverslips with Triton X-100, the fibrillar extracellular deposits on the culture surface which stained positively for fibronectin were especially well seen (Fig 3*C*). In colonies of

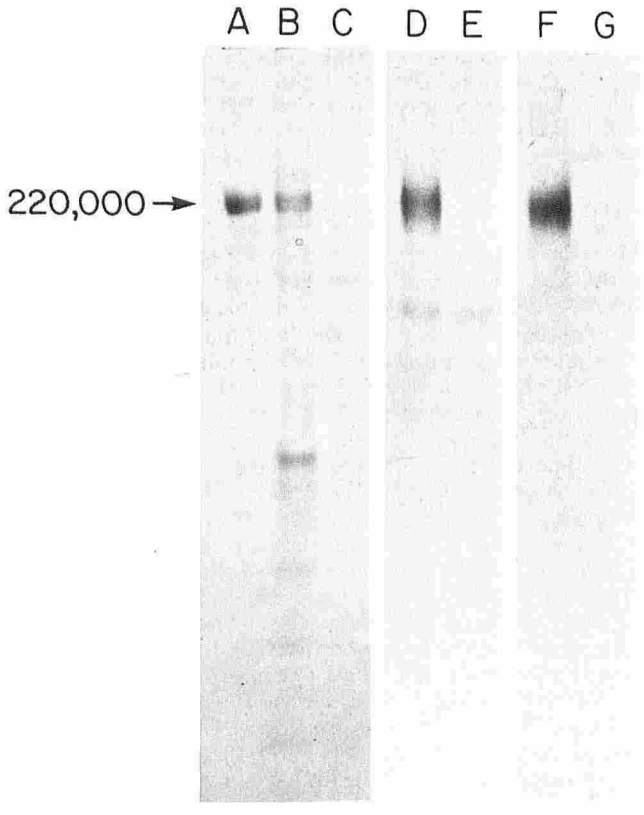

FIG 1. Synthesis of fibronectin by cultured keratinocytes. Cells in medium with 2.5% fetal bovine serum were labeled with [^{35}S]methionine as in *Methods and Materials*. Medium was collected, centrifuged (20,000 g × 15 min) at 4°C, made 2 mM in iodoacetamide, N-ethyl maleimide, and phenyl methyl sulfonyl fluoride and either extracted with 100 μl of a 50% slurry of gelatin-Sepharose or immunoprecipitated with affinity-purified anti-fibronectin antibody as in *Methods and Materials*. Autoradiograms were made from the Coomassie blue-stained gels. *A–C*, Coomassie blue-stained gel showing fibronectin (20 μg, *A*) and material released from either gelatin-Sepharose (*B*) or unsubstituted Sepharose (*C*). *D–G*, autoradiograms of [^{35}S]methionine-labeled material released from gelatin-Sepharose (*D*) or unsubstituted Sepharose (*E*) or of material immunoprecipitated by antifibronectin (*F*) or by control rabbit IgG (*G*).

cells, the fibrillar arrays of material staining for fibronectin were most clearly visualized at the plane of the glass coverslip upon which the cells were cultured (Fig 3*D*). At higher planes above the substratum, striking intracellular staining of the keratinocytes was also seen (Fig 3*E*). Controls (Fig 3*F*) treated with normal rabbit IgG showed no extracellular staining in high or low Ca^{++} and negligible perinuclear nonspecific staining.

The patterns of fibronectin in and around cells differed at physiologic and reduced Ca^{++} concentrations. At higher Ca^{++} concentrations (1.1 mM) the location of cells and colonies correlated well when compared by immunofluorescence (Fig 4*A*) and phase contrast (Fig 4*B*) microscopy. Marked staining of fibronectin in a perinuclear distribution was seen; this was thought to be intracellular, since it was not present unless cells were seen by phase contrast on the glass surface, but the extracellular staining persisted as in Fig 3*C*. At reduced Ca^{++} concentrations, however, very little intracellular fibronectin was seen (Fig 4*C*), and the patterns correlated poorly when compared by immunofluorescence (Fig 4*C*) and phase contrast (Fig 4*D*) microscopy, indicating that some cells no longer present had laid down fibronectin and that others appeared to have laid down little. Melanocytes, which could be recognized by the presence of melanin granules and long processes, consistently failed to stain for fibronectin. The melanocyte spanning the distance between two colonies in Fig 4*B* does not stain in Fig 4*A*.

Although cultures were free of contaminating fibroblasts by morphologic criteria, we determined that the cells producing patterns of fibronectin which we ascribed to keratinocytes were not 3T3 cells or human fibroblasts by staining the cultures with antikeratin antibody. It was important to exclude contamination by fibroblasts to assure that fibronectin secreted by a contaminating cell did not bind to keratinocytes and appear to originate from them. All cells in our keratinocyte cultures that stained with anti-fibronectin antibody (Fig 5*A*) stained with antikeratin antibody (Fig 5*B*), and no cells failing to stain for keratin were observed. At reduced Ca^{++} concentrations patterns of fibronectin (Fig 5*C*) and keratin (Fig 5*D*) staining reflected

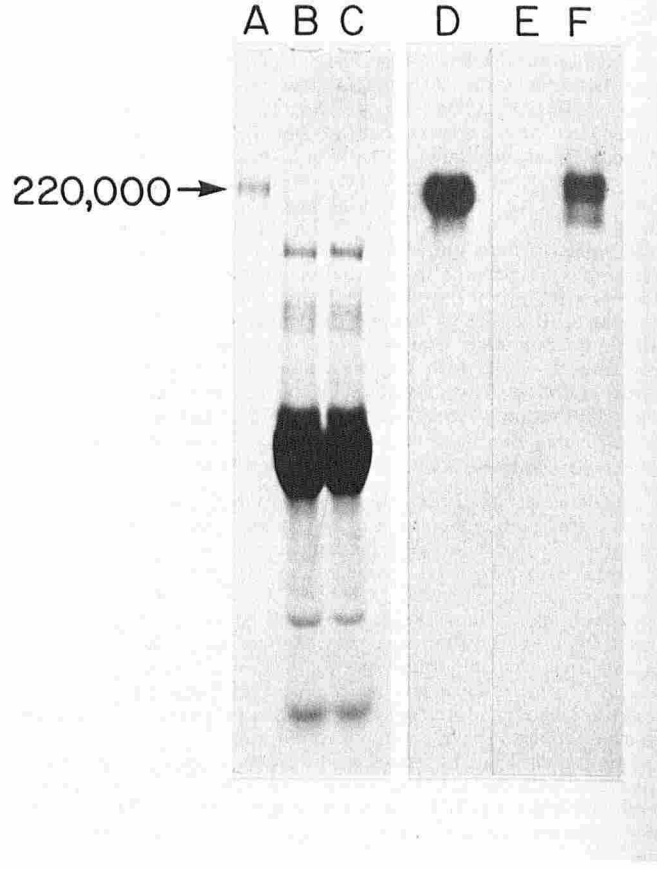

FIG 2. Immunoblot of fibronectin in normal and gelatin-Sepharose extracted fetal bovine serum. Fibronectin purified from human plasma (*A*, *D*) or fetal bovine serum (depleted of fibronectin by passage twice over gelatin-Sepharose (*B*, *E*) or unextracted (*C*, *F*)) was electrophoresed and stained with Coomassie blue (*A–C*) or transferred to nitrocellulose and developed as in *Methods and Materials* using affinity-purified anti-fibronectin antibody at 1:100 dilution. *A*, Fibronectin, 10 μg. *B*, Extracted fetal bovine serum, 15 μl of 1:30 dilution in water. *C*, Unextracted serum at same dilution. *D–F*, immunoblots corresponding to *A–C* but using only 0.25 μg of fibronectin in *D*. *E*, Extracted serum. *F*, Unextracted serum. The lower band in *F* is a proteolytic product from the higher-molecular-weight dimer, which runs as a doublet.

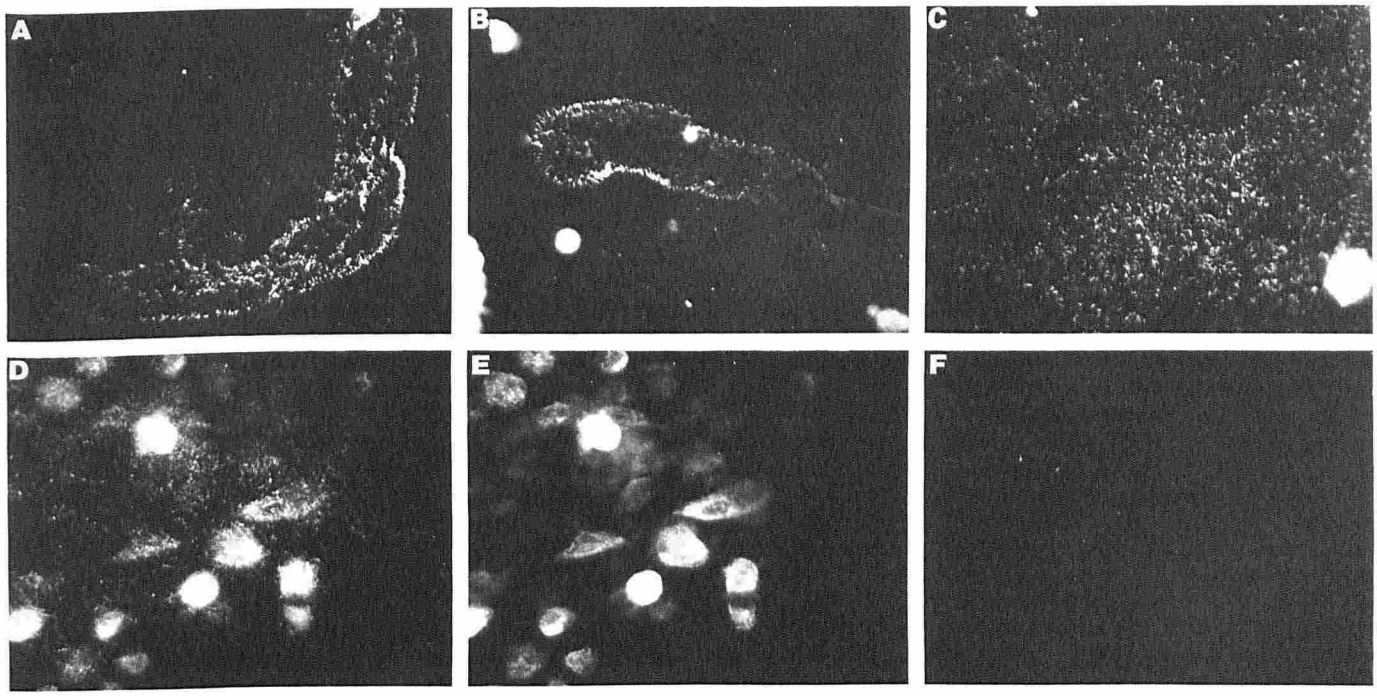

FIG 3. Immunofluorescence showing location and pattern of fibronectin deposited by keratinocytes. *A and B*, Asymmetric patterns of fibronectin left by keratinocytes at reduced Ca^{++} concentrations (× 160). *C*, Fibrillar deposits on coverslip deposited by keratinocytes no longer present (× 320). *D and E*, Demonstration of the location of fibronectin by focusing either beneath the cells (*D*) or at the plane of the cells (*E*) (× 160). *F*, Control section showing lack of nonspecific staining (× 160). *A* and *B* were grown in Joklik's medium with 0.07 mM Ca^{++} and 2.5% fibronectin-depleted fetal bovine serum. *C–F* were grown in ½ Hams F-12, ½ DME with 1.1 mM Ca^{++} and 5% fibronectin-depleted fetal bovine serum.

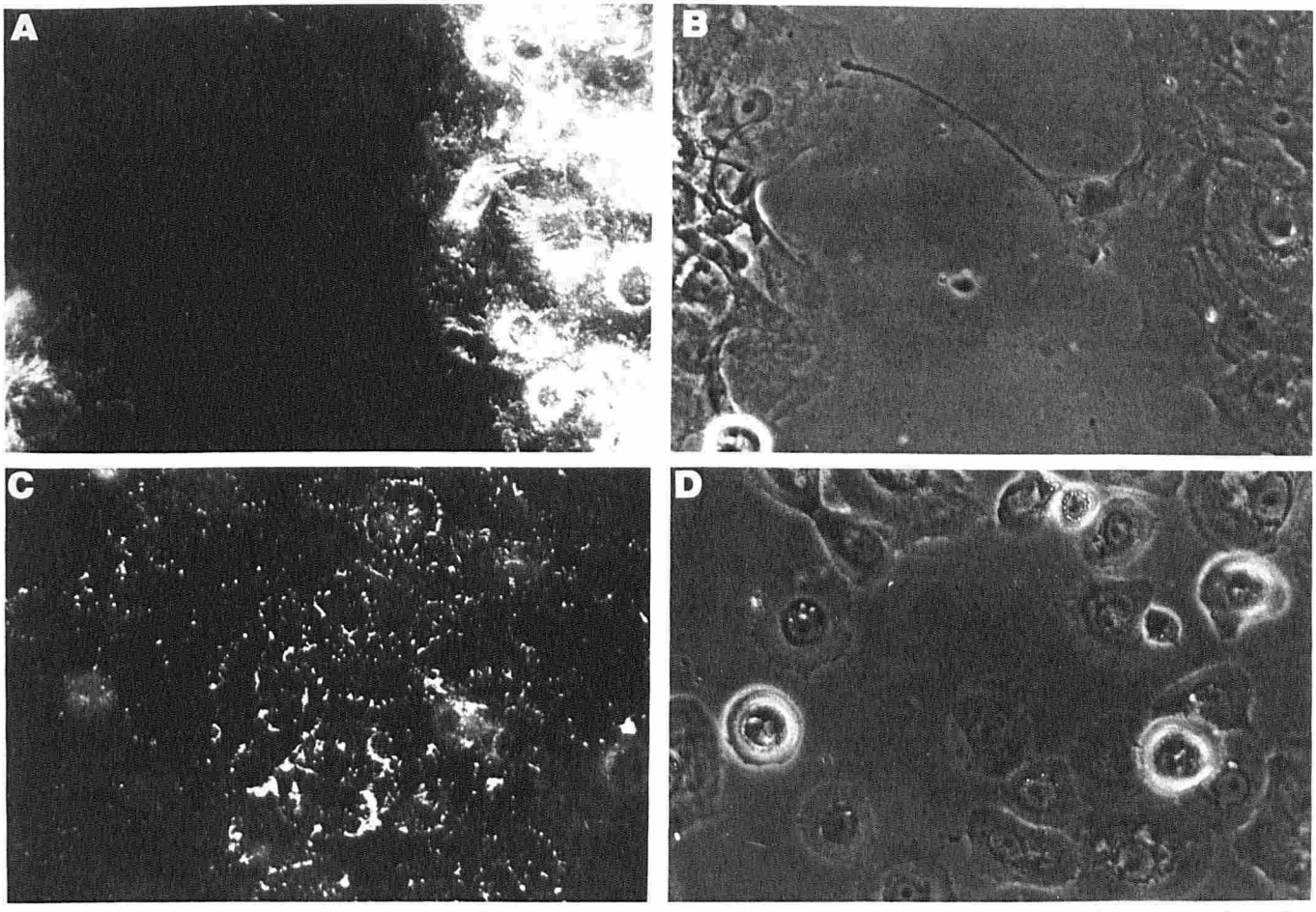

FIG 4. Relationship of fibronectin pattern to cells at normal or reduced Ca^{++} concentrations. *A and B*, Colonies of keratinocytes in medium with 1.1 mM Ca^{++} observed by immunofluorescence (*A*) or phase contrast (*B*) optics (× 120). *C and D*, Keratinocytes plated and grown at 0.07 mM Ca^{++} in Joklik's medium under immunofluorescence (*C*) or phase contrast (*D*) optics (× 200). Fibronectin-depleted serum concentrations as in Fig 3.

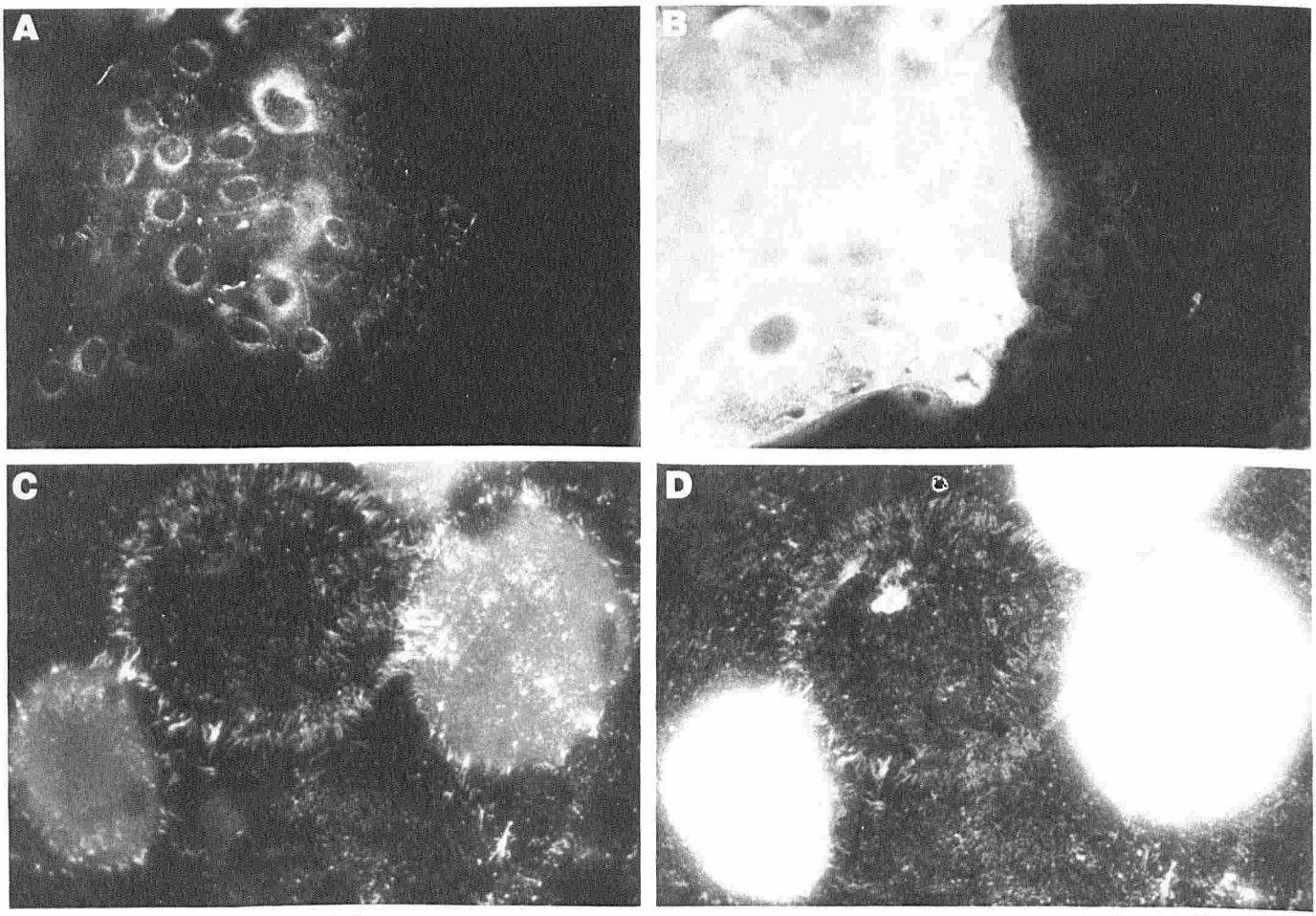

FIG 5. Comparison of keratin and fibronectin immunofluorescence in keratinocytes plated and grown at normal and reduced Ca^{++} concentrations. Cells were treated sequentially with rabbit antifibronectin, fluorescein antirabbit conjugate, rabbit antikeratin, and rhodamine antirabbit conjugate. This stains fibronectin with fluorescein and keratin with both fluorescein and rhodamine. *A and B*, Keratinocytes grown at 1.1 mM Ca^{++} stained for fibronectin with fluorescein (*A*) or for fibronectin and keratin with rhodamine (*B*) (× 120). *C and D*, Keratinocytes grown at 0.07 mM Ca^{++} in Joklik's medium stained for fibronectin (*C*) or fibronectin and keratin (*D*) (× 604).

the altered morphology of the cells, and fibronectin could be shown to be mostly extracellular, since cells that became detached left behind a matrix containing most of the fibronectin seen in association with intact cells (Fig 5*C,D*). In these double-labeling studies, both fibronectin and keratin are stained with rhodamine (since rabbit antibodies and rhodamine-labeled antirabbit IgG were used) but only fibronectin is stained with fluorescein (because of the sequence of stains used, as noted in the legend to Fig 5).

DISCUSSION

The production of fibronectin has been examined in several types of epithelial cells. Chen et al [8] demonstrated fibronectin by immunofluorescence in primary cultures of rat liver and hamster kidney cells and noted that fibrillar networks nearly disappeared after multiple passages and were never as extensive as those around fibroblasts. Smith et al [32] demonstrated fibronectin production in mammary and intestinal epithelium and in a mammary carcinoma by metabolic labeling. Others have also demonstrated fibronectin in cultures of rat [9] and human [10] mammary cells and in liver cells [5,6], which can synthesize plasma fibronectin [6]. Quaroni et al [7] showed that fibronectin was synthesized by a cell line derived from rat intestinal epithelium. Alitalo et al [18] used metabolic labeling to show that keratinocytes grown with a 3T3 cell feeder layer produced fibronectin after the feeder layer had been removed.

In a paper describing keratinocyte matrix proteins, Kariniemi et al [19] showed similar fibronectin synthesis using keratinocytes prepared from blistered epidermis and showed immunofluorescent photographs of fibronectin. Peehl and Stanbridge [20] showed a photograph of keratinocyte fibronectin in a paper on somatic cell hybrids. None of these authors used fibronectin-free medium to assure that the material stained was not derived from the culture media.

Fibronectin from fetal bovine serum can become incorporated into the matrix of cultured cells [14], and fibronectin secreted by cultured cells can be incorporated into the matrix of another cell type [33]. Furthermore, human plasma fibronectin has been shown to bind to cultured human skin fibroblasts and to become incorporated into the detergent-insoluble matrix composed primarily of cellular fibronectin [15]. The significance of this phenomenon is increased by the finding that human plasma fibronectin can bind to mouse tissues when injected in vivo [11]. It is essential, therefore, to use fibronectin-depleted medium to study fibronectin deposition in cultured cells.

In our studies, keratinocytes grown in fibronectin-depleted medium displayed intra- and extracellular fibronectin. This material differed from fibroblast fibronectin in its location exclusively adjacent to the glass substratum, as described by Mosher in other non-fibroblastic cell types [4]. Human fibroblasts have been shown to produce insoluble fibronectin first in this location and later in a pericellular matrix [4]. Fibroblast fibronectin was readily demonstrated in unextracted cells in our studies, but keratinocyte fibronectin was only partially revealed unless cells were extracted by solvents such as methanol or acetone or by detergents such as Triton X-100 (data not shown). Similar patterns of fibronectin were demonstrated

in tumorigenic keratinocyte lines PAM 212 (mouse) and A-431 (human) (data not shown).

A novel feature of the fibronectin produced by keratinocytes is the presence of occasional asymmetric patterns suggesting movement. Other techniques will be required to document this possibility. Several models for the possible role of fibronectin in directional movement of cells have been suggested by Hynes [1], including one in which cells lay down "tracks" of fibronectin in response to a vectorial stimulus and then travel along these tracks. Tracking of cells along a path defined by residual fibronectin was demonstrated in fibroblasts with colloidal gold, which was used to follow the path of motile cells [12]. The additional 3-dimensional pericellular fibronectin matrix produced by fibroblasts may have functions different from those of substrate-attached fibronectin in epithelial cells.

The pattern and distribution of fibronectin was altered in cells cultured in medium with 0.07 mM Ca^{++} using Joklik's medium in combination with fetal bovine serum depleted of Ca^{++} by Chelex-100. Intracellular fibronectin was not seen, and the fibrillar pattern was more difficult to find. These cells also failed to stratify, were rounded, and were less firmly attached to the plastic surface. Since keratinocytes growing at reduced Ca^{++} concentrations are only loosely attached to the culture surface, it is possible that this is related to their less extensive anchoring with fibronectin fibrils. The less complex extracellular fibronectin matrix and the lack of intracellular fibronectin suggest decreased synthesis, but estimates of fibronectin production at different Ca^{++} concentrations will require quantitative techniques.

Keratinocytes have been shown to attach to type IV collagen without a soluble plasma factor [34], and attachment of a mouse epithelial line to type IV collagen was enhanced by laminin [35], a basement membrane protein. Nevertheless, fibronectin may have a role in keratinocyte attachment, spreading, and movement. First, Stenn et al [36] have shown that fibronectin can act as a "spreading factor" for guinea pig epidermal cells under certain conditions. Second, freshly prepared human keratinocytes attach and grow on fibronectin-coated surfaces better than on collagen-coated or uncoated tissue culture plastic [37]. Finally, fibronectin is part of the temporary matrix for keratinocytes in a healing wound but later disappears [38]. Whether fibronectin can be produced by keratinocytes in vivo under certain conditions as in wound healing or morphogenesis is unknown.

Note added in proof: Gibson et al [39] have recently demonstrated insoluble fibronectin in rat keratinocyte cultures using antibody absorbed with fetal bovine serum.

REFERENCES

1. Hynes RO: Fibronectin and its relation to cellular structure and behavior, Cell Biology of the Extracellular Matrix. Edited by ED Hay. New York, Plenum Press, 1982, pp 295–334
2. Hynes RO, Yamada KM: Fibronectins: multifunctional modular glycoproteins. J Cell Biol 95:369–377, 1982
3. Ruoslahti E, Engvall E, Hayman EG: Fibronectin: current concepts of its structure and function. Collagen Research 1:95–128, 1981
4. Mosher DF: Fibronectin. Prog Hemost Thromb 5:111–151, 1980
5. Voss B, Allam S, Rauterberg J, Ullrich K, Gieselmann V, von Figura K: Primary cultures of rat hepatocytes synthesize fibronectin. Biochem Biophys Res Commun 90:1348–1354, 1979
6. Tamkun JW, Hynes RO: Plasma fibronectin is synthesized and secreted by hepatocytes. J Biol Chem 258:4641–4647, 1983
7. Quaroni A, Isselbacher KJ, Ruoslahti E: Fibronectin synthesis by epithelial crypt cells of rat small intestine. Proc Natl Acad Sci USA 75:5548–5552, 1978
8. Chen LB, Maitland N, Gallimore PH, McDougall JK: Detection of the large external transformation-sensitive protein on some epithelial cells. Exp Cell Res 106:39–46, 1977
9. Warburton MJ, Head LP, Rudland PS: Redistribution of fibronectin and cytoskeletal proteins during the differentiation of rat mammary tumor cells *in vitro*. Exp Cell Res 132:57–66, 1981
10. Asch BB, Kamat BR, Burstein NA: Interactions of normal, dysplastic and malignant mammary epithelial cells with fibronectin *in vivo* and *in vitro*. Cancer Res 41:2115–2125, 1981
11. Oh E, Pierschbacher M, Ruoslahti E: Deposition of plasma fibronectin in tissues. Proc Natl Acad Sci USA 78:3218–3221, 1981
12. Ali IA, Haynes RO: Effects of LETS glycoprotein on cell motility. Cell 14:439–446, 1978
13. Pennypacker JP, Hassell JR, Yamada KM, Pratt RM: The influence of an adhesive cell surface protein on chondrogenic expression in vitro. Exp Cell Res 121:411–415, 1979
14. Hayman EG, Ruoslahti E: Distribution of fetal bovine serum fibronectin and endogenous rat cell fibronectin in extracellular matrix. J Cell Biol 83:255–259, 1979
15. McKeown-Longo PJ, Mosher DF: Binding of plasma fibronectin to cell layers of human skin fibroblasts. J Cell Biol 97:466–472, 1983
16. Stenman S, Vaheri A: Distribution of a major connective tissue protein, fibronectin, in normal human tissues. J Exp Med 147:1054–1063, 1978
17. Boselli JM, Macarak EJ, Clark CC, Brownell AG, Martinez-Hernandez A: Fibronectin: its relationship to basement membranes. Collagen Research 5:391–404, 1981
18. Alitalo K, Kuismanen E, Myllya R, Kiistalo U, Aski-Seljavaara S, Vaheri A: Extracellular matrix proteins of human epidermal keratinocytes and feeder 3T3 cells. J Cell Biol 94:497–505, 1982
19. Kariniemi A-L, Lehto V-P, Vartio T, Virtanen I: Cytoskeleton and pericellular matrix organization of pure adult human keratinocytes cultured from suction-blister roof epidermis. J Cell Sci 58:49–61, 1982
20. Peehl DM, Stanbridge EJ: Characterization of human keratinocyte X HeLa somatic cell hybrids. Int J Cancer 27:625–635, 1981
21. Rheinwald JG, Green H: Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331–344, 1975
22. O'Keefe E, Battin T, Payne R Jr: Epidermal growth factor receptor in human epidermal cells: direct demonstration in cultured cells. J Invest Dermatol 78:482–487, 1982
23. Brennan JK, Mansky J, Roberts G, Lichtman MA: Improved methods for reducing calcium and magnesium concentrations in tissue culture medium. In Vitro 11:354–360, 1975
24. Hayashi M, Yamada KM: Divalent cation modulation of fibronectin binding to heparin and to DNA. J Biol Chem 257:5263–5267, 1982
25. Parikh I, March S, Cuatrecasas P: Topics in the methodology of substitution reactions with agarose. Meth Enzymol 34:77–102, 1974
26. Fairbanks G, Steck TL, Wallach DFH: Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606–2617, 1971
27. O'Keefe EJ, Battin TK, Bennett V: Proteolytic domains of the epidermal growth factor receptor of human placenta. J Supramolec Struct Cell Biochem 15:15–27, 1981
28. Towbin H, Staehlin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Proc Natl Acad Sci USA 76:4350–4354, 1979
29. Burnett, WN: "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 112:195–203, 1981
30. Hunter WM, Greenwood FC: Preparation of iodine-131 labeled human growth hormone of high specific activity. Nature 194:495–496, 1962
31. Choi MC, Hynes RO: Biosynthesis and processing of fibronectin in NIL.8 hamster cells. J Biol Chem 254:12050–12055, 1979
32. Smith HS, Riggs JL, Mossesson MW: Production of fibronectin by human epithelial cells in culture. Cancer Res 39:4138–4144, 1979
33. Hayman EG, Engvall E, Ruoslahti E: Concomitant loss of cell surface fibronectin and laminin from transformed rat kidney cells. J Cell Biol 88:352–357, 1981
34. Murray JC, Stingl G, Kleinman HK, Martin GR, Katz SI: Epidermal cells adhere preferentially to type IV (basement membrane) collagen. J Cell Biol 80:197–202, 1979
35. Terranova VP, Rohrbach DH, Martin GR: Role of laminin in the attachment of PAM 212 (epithelial) cells to basement membrane collagen. Cell 22:719–728, 1980
36. Stenn KS, Madri JA, Tinghitella T, Terranova V: Multiple mechanisms of disassociated epidermal cell spreading. J Cell Biol 96:63–67, 1983
37. Gilchrest BA, Calhoun JK, Maciag T: Attachment and growth of human keratinocytes in a serum-free environment. J Cell Physiol 112:197–206, 1982
38. Clark RAF, Lanigan JM, Dellapelle P, Manseau E, Dvorak HF, Colvin RB: Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization. J Invest Dermatol 79:264–269, 1982
39. Gibson WT, Couchman JR, Badley A, Saunders HJ, Smith CG: Fibronectin in cultured rat keratinocytes: distribution, synthesis, and relationship to cytoskeletal proteins. Eur J Cell Biol 30:205–213, 1983