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Spreading and Enhanced Motility of Human Keratinocytes on Fibronectin

EDWARD J. O'KEEFE, M.D., RICHARD E. PAYNE, JR., B.S., NANCY RUSSELL, B.S., AND DAVID T. WOODLEY, M.D.

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

Soluble human plasma fibronectin or collagen types I or IV, when preincubated with tissue culture plastic dishes, were effective spreading agents for cultured human keratinocytes and increased spreading in a time- and concentration-dependent manner. Spreading on fibronectin, but not on type IV collagen, was inhibited by antifibronectin; therefore, the contribution of fibronectin to the spreading activity of the natural matrix produced by keratinocytes could not be determined using antifibronectin. Fibronectin mediated spreading at both high (1.1 mM) and low (0.1 mM) Ca^{++} concentrations, and spreading was not altered by cycloheximide. Insoluble fibronectin deposited by keratinocytes correlated with phagokinetic tracks on particulate gold salts, and added fibronectin, as well as type I collagen and type IV collagen, enhanced motility of keratinocytes. These studies show that production of fibronectin and responsiveness to it are similar in fibroblasts and keratinocytes and demonstrate that fibronectin can act as a matrix factor for keratinocytes.

The role of fibronectin in the biology of keratinocytes is uncertain. For example, guinea pig epidermal cells showed enhanced attachment to type IV collagen in comparison with other collagens, and this was unaffected by fibronectin [1]. Furthermore, laminin, but not fibronectin, has been shown to increase attachment of PAM 212 cells, a mouse keratinocyte line, to collagen [2]. These findings suggested that fibronectin, an attachment factor for fibroblasts, had no effect on attachment of keratinocytes. Furthermore, fibronectin is not found within normal epidermis [3]. Because of these findings, it has been suggested that fibronectin has specificity for fibroblasts and some other cell types and does not interact significantly with epithelium or epithelial cells [4]. Its presence in mature cutaneous basement membrane, which is elaborated largely by the epithelium, is debated [5] although it has been demonstrated in fetal rat skin [6]. Nevertheless, several types of epithelial cells produce fibronectin in culture [7–11], and we [12] and others [13–15] have demonstrated that soluble and insoluble fibronectins are secreted by keratinocytes. Gilchrest et al have shown enhanced attachment and growth of keratinocytes on fibronectin [16], and Stenn *et al.* have recently described the ability of fibronectin to enhance the spreading of guinea pig keratinocytes [17]. We have now investigated the role of fibronectin and some other matrix components in spreading and movement of cultured human keratinocytes. The findings indicate that the cells respond similarly to fibronectin and to collagen and therefore further support the role of fibronectin as a matrix factor for keratinocytes.

MATERIALS AND METHODS

Cell Culture

Human foreskin keratinocytes were prepared and cultured by the method of Rheinwald and Green [18] as previously described [12]. 3T3 cells were removed with calcium-free Hanks' buffered salt solution (HBSS) containing 0.02% EDTA, and keratinocytes were harvested with trypsin and subcultured in medium composed of 50% 3T3 cell conditioned medium [made up of Dulbecco-Vogt modification of Eagle's medium (DME) with 5% fetal bovine serum (FBS)] and 50% Ham's F-12. Medium was supplemented with insulin (5 μg/ml), hydrocortisone (0.4 μg/ml), transferrin (5 μg/ml), cholera toxin (5 ng/ml), and epidermal growth factor (EGF) (5 ng/ml). Keratinocytes cultured in this fashion were shown to be free of fibroblasts [12].

Spreading Assays

Cells subcultured as noted above were harvested with trypsin and plated at 5×10^4 per 35-mm dish on untreated or coated tissue culture dishes. To coat tissue culture surfaces, we added 2 ml HBSS to a 35-mm dish and then added up to 40 μg of type IV collagen, type I collagen, or fibronectin. After 30 min at 37°C we removed the HBSS and added nutrient medium and cells. Medium for spreading assays was either medium A, 50% DME/50% Ham's F-12 (1.1 mM Ca^{++}), or medium B, MCDB 153 with added $CaCl_2$ (0.1 mM Ca^{++}). Supplements in medium A were hydrocortisone (0.4 μg/ml), insulin (5 μg/ml), EGF (5 ng/ml), transferrin (5 μg/ml), and cholera toxin (5 ng/ml). Medium B contained hydrocortisone, insulin, and transferrin as above plus 0.1 mM ethanolamine and phosphoethanolamine. Type IV collagen was purified from EHS sarcoma [19], and has been characterized previously [20]. Type IV collagen from pepsin-digested placenta purchased from Sigma was also effective as a spreading agent.

In the standard assay, dishes were washed gently twice with HBSS after 4 h at 37°C and adherent cells fixed in 3% formaldehyde in Dulbecco's phosphate-buffered saline (PBS). The percentage of spread cells was determined by counting dishes in triplicate sets of 100 cells each and determining the mean ± SEM. Spread cells were identified under phase contrast microscopy as dark cells with polarity or radially distributed lamellipodia or a continuous apron and a visible nucleus. Unspread cells were highly refractile and round.

Assays of Motility

Coverslips were plated with particulate gold salts according to Albrecht-Buehler [21]. Briefly, 1.8 ml $HAuCl_4$ (14.5 mM) was added to 6 ml of 36.5 mM Na_2CO_3 and diluted with 11 ml H_2O. The solution was heated just to boiling and 1.8 ml of 0.1% formaldehyde was added. The solution, which now contained gold particles, was added to dishes containing coverslips which had been dipped in 1% bovine serum albumin and drained, then in 100% ethanol, and dried. After 45 min, the solution was aspirated and replaced with PBS. Cells were plated on the coverslips in nutrient medium A or B within 24 h of preparation of the coverslips and allowed to attach, spread, and migrate. After various times the dishes were washed gently to remove nonadherent cells, fixed in 3% formaldehyde in PBS, and cells on the coverslips were photographed in the dish under dark-field optics. In some experiments coverslips were processed for immunofluorescence, mounted, and photographed. Gold particles on the same slide were photographed

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Reprint requests to: Edward J. O'Keefe, M.D., Department of Dermatology, University of North Carolina, School of Medicine, 137 North Carolina Memorial Hospital, Chapel Hill, North Carolina, 27514.

Abbreviations:
DME: Dulbecco-Vogt modification of Eagle's medium
EGF: epidermal growth factor
FBS: fetal bovine serum
HBSS: Hanks' buffered salt solution
PBS: Dulbecco's phosphate-buffered saline

under phase contrast optics. In other experiments coverslips with gold particles were incubated with matrix proteins to coat coverslips as under *Spreading Assays* above before cells were added.

Immunofluorescence

Cells on coverslips were fixed in 3% formaldehyde in PBS, washed, extracted in 1% Triton X-100, and stained as described [12] using affinity-purified rabbit antihuman plasma fibronectin followed by fluorescein-conjugated goat antirabbit immunoglobulin. Photographs were made on a Leitz Orthoplan microscope with epifluorescence optics using Kodak Tri-X film developed in Diafine (Acufine, Inc., Chicago, Illinois).

Materials

Culture media were from Gibco Laboratories, Grand Island, New York (Ham's F-12, DME, FBS, HBSS) or KC Biological, Lenexa, Kansas, (MCDB 153). Supplements (insulin, hydrocortisone, transferrin, ethanolamine, phosphoethanolamine), bovine serum albumin, and placental type IV collagen were from Sigma, St. Louis, Missouri. EGF was purified according to Savage and Cohen [22]. Plastic tissue culture ware and Vitrogen were from Flow Laboratories, McLean, Virginia. Chemicals were from Fisher Scientific, Pittsburgh, Pennsylvania. Antifibronectin antibody was purified on fibronectin-Sepharose as previously described [12]. Rabbit IgG and fluorescein-labeled antirabbit IgG were from Cappel Laboratories, West Chester, Pennsylvania. Fibronectin was purified from human plasma according to Hayashi and Yamada [23]. Type I collagen was also prepared from rat tail tendon [24]. Purified collagens [20], laminin [20], and fibronectin [12] have been characterized previously.

RESULTS

Spreading of Keratinocytes by Fibronectin or Collagen

Small amounts of type IV collagen or human plasma fibronectin added to buffered saline solutions such as HBSS attached to culture dishes [25] and were effective as spreading agents after brief incubations (30 min, 37°C) without drying or UV irradiation. After removal of the solution to which the matrix protein had been added, cells were added to the dishes in nutrient medium and became maximally spread after several hours. As shown in Fig 1, spreading was enhanced after addition of 5–10 μg of fibronectin and was maximal with 40 μg or more. Similar results were obtained with type IV collagen; thus, fibronectin was as effective as type IV collagen as a spreading agent for cultured human keratinocytes. Two preparations of type I collagen (from rat tail tendon or bovine skin) were also very effective spreading factors, and the effect of all 3 agents was not altered by cycloheximide (Table I).

Mosher's laboratory has described specific receptors for fibronectin [26]; this finding suggests that spreading by fibronectin or collagen may be mediated through cell surface receptors. To determine whether we could differentiate spreading by type IV collagen or fibronectin, we tested the effect of antifibronectin antibody on spreading induced by fibronectin or type IV collagen. First, using small amounts of fibronectin, we found that antifibronectin added with cells to fibronectin-coated dishes completely prevented spreading, but control IgG was ineffective. Serum slightly augmented spreading, but the effect of antifibronectin was apparent either in the presence or absence of serum (*A* in Table II). Second, the effect of antifibronectin was examined on cells plated on fibronectin or type IV collagen to determine the specificity of action of the antibody. As shown in Table II (*B*), antifibronectin reduced fibronectin-induced spreading to control levels but did not alter spreading by type IV collagen. We concluded that the spreading activities of fibronectin and type IV collagen are mediated differently, probably by the receptor for these proteins, and not by a common, nonspecific mechanism.

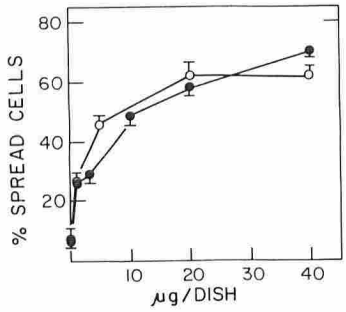

FIG 1. Spreading of keratinocytes by fibronectin or type IV collagen. Human plasma fibronectin or type IV collagen was incubated with dishes as in *Materials and Methods* and plated with 5×10^4 cultured cells in medium A. After 4 h at 37°C dishes were washed twice with HBSS, fixed with formalin, and scored for spreading in triplicate under phase-contrast microscopy. ● = Collagen IV; ○ = fibronectin.

TABLE I. *Spreading by fibronectin or collagen*[a]

	Addition	Percent spread cells	
	Cycloheximide	−	+
Exp. I[b]	Matrix		
	None	4 ± 1	3 ± 1
	Coll I (Vitrogen)	94 ± 0	94 ± 2
	Coll I (rat tail tendon)	85 ± 6	87 ± 2
	Coll IV	86 ± 5	96 ± 1
	Fibronectin	88 ± 2	88 ± 1
Exp. II[c]	None	11 ± 3	7 ± 2
	Coll IV (3 μg)	90 ± 1	87 ± 2
	Coll IV (40 μg)	95 ± 2	94 ± 4

[a] Results are mean ± SEM of triplicate determinations.
[b] Dishes were incubated with or without matrix factors (30 μg) in HBSS (30 min, 37°C) and then plated with cells in medium A with or without cycloheximide (25 μg/ml). After 4 h at 37°C cells were assayed for spreading. *Coll* = collagen.
[c] Dishes were preincubated with or without type IV collagen and assayed as in (*b*).

TABLE II. *Antifibronectin specifically prevents fibronectin-induced spreading*

	Additions	Percent spreading[a]	
		−Serum	+Serum
A	None	3 ± 1	6 ± 3
	FN	18 ± 4	27 ± 3
	FN + antiFN	1 ± 0	1 ± 0
	FN + IgG	26 ± 6	27 ± 6
		IgG	AntiFN
B	None	9 ± 4	10 ± 3
	FN	50 ± 7	10 ± 3
	Coll IV[b]	68 ± 6	65 ± 7

After 35-mm dishes were treated with or without fibronectin (*FN*) or type IV collagen (*Coll IV*), 0.8 ml medium A containing 200 μg normal rabbit IgG or affinity-purified rabbit antiFN was added. *A*, Dishes were treated with or without 5 μg FN. After 30 min at 37°C, 5×10^4 keratinocytes were added in 1.2 ml medium A with or without 5% heat-inactivated FBS and assayed for spreading as in *Materials and Methods*. *B*, Dishes were treated with or without 30 μg FN or Coll IV, and medium contained no serum.
[a] Mean ± SEM of triplicate determinations.
[b] From EHS tumor.

The concentration of Ca^{++} in the medium is known to influence stratification and proliferation of keratinocytes [27] as well as display of some cell surface receptors [28]. Since spreading presumably depends on cell surface binding of added matrix proteins, it might be affected by Ca^{++} concentration. As shown in Table III, keratinocytes were able to spread on matrix-coated surfaces at both high (1.1 mM) and low (0.1 mM) Ca^{++} concentrations. Also, in agreement with the data of Stenn et al on fresh guinea pig epidermal cells [17], the spreading activity of fibronectin and type IV collagen was not prevented by cycloheximide. EGF, which is a growth factor for keratinocytes and may make colonies appear more flattened, was incubated

TABLE III. *Spreading of keratinocytes independent of Ca^{++} concentration and cycloheximide*[a]

Additions	1.1 mM Ca^{++}[b] Cycloheximide		0.1 mM Ca^{++}[c] Cycloheximide	
	−	+	−	+
None	2 ± 1	1 ± 1	4 ± 2	2 ± 1
FN	76 ± 5	65 ± 4	93 ± 3	89 ± 2
Coll IV	87 ± 5	84 ± 3	94 ± 4	96 ± 4
EGF	3 ± 1	2 ± 1	4 ± 2	2 ± 1
Laminin[d]	2 ± 3	6 ± 2	—	—

Keratinocytes (5×10^4 cells) were plated on dishes coated with or without fibronectin (*FN*) (20 μg) or type IV collagen (*Coll IV*) (20 μg) as in *Materials and Methods.* Cells were incubated with or without epidermal growth factor (*EGF*) (20 ng/ml), laminin (20 μg/ml), or cycloheximide (25 μg/ml) for 4 h, 37°C, and assessed for spreading.

[a] Results given in mean ± SEM of triplicate determinations.

[b] Medium A.

[c] Medium B.

[d] Purified from EHS sarcoma.

TABLE IV. *Enhancement of spreading by matrix deposited by keratinocytes*[a]

Treatment of culture[b]	Percent spreading at 4 h[d]	
Dispase	42 ± 4	
Scraping	43 ± 3	
Trypsin	22 ± 4	
1% Triton X-100	39 ± 4	
Matrix[c]	4 Hours	24 Hours
None	3 ± 3	42 ± 8
Coll IV	56 ± 5	74 ± 4

[a] Human keratinocytes were subcultured without feeder layers as in *Materials and Methods.*

[b] After cultures became confluent, cell layers were removed by Dispase (1 unit/ml) (Boehringer Mannheim, Indianapolis, Indiana) by scraping with a Teflon policeman, by treatment with 0.25% trypsin (1:250, Gibco, Grand Island, New York), or by incubation with 1% Triton X-100 in water and several washes to remove the detergent. Dishes were then plated with 5×10^4 cells in medium A for 4 h as in the standard spreading assay.

[c] Dishes were incubated with or without 20 μg type IV collagen (*Coll IV*) (30 min, 37°C) or 10% FBS in medium A (2 h, 37°C), washed twice with HBSS, and plated in parallel with (*b*) and assayed for spreading after 4 or 24 h.

[d] Mean ± SEM of triplicate determinations.

with the cells and had no spreading activity at 4 h (Table III). Laminin (either purified from EHS sarcoma or purchased from BRL, Bethesda, Maryland) failed to increase spreading when added either before cells and medium in an attempt to coat the surface or with medium (Table III).

We have noted that if keratinocytes were trypsinized and then plated on the dish on which they were originally grown, spreading occurred rapidly, but attempts to standardize preparations of plates were unsuccessful. We suspected that a matrix material produced by the cells was responsible for spreading, and some of this material apparently remained on the dish when cells were removed by trypsin, scraping, nonionic detergent, or Dispase (Table IV). It was not as effective as purified matrix factors, however, in enhancing spreading, and results were variable. Fibronectin from FBS was probably not responsible for this activity, since antifibronectin was not able to prevent the spreading (not shown). As antifibronectin cannot prevent spreading induced by type IV collagen (Table II) and probably by other spreading factors, another spreading agent in the matrix (e.g., collagen) may have been responsible for spreading on the natural matrix.

Role of Spreading Factors in Movement of Keratinocytes

Because fibronectin has been implicated in fibroblast motility [29], we were interested to find out whether fibronectin and other matrix factors can affect motility of keratinocytes. We have previously described the laying down of tracks of fibronectin (seen by immunofluorescence) by human keratinocytes in culture [12]. These tracks suggested paths of movement by the cells. To document the association of these tracks with movement, we used the technique of Albrecht-Buehler [21]. Colloidal gold salts were deposited on the surfaces of glass coverslips, and cultured keratinocytes were plated on the particulate salts. When cells migrate on this surface, the displacement of particles is the result of movement of cells and phagocytosis of particles; they have therefore been called "phagokinetic" tracks [21]. Cells were fixed with formalin and the coverslips were examined by phase microscopy to determine the location of the particles and examined by immunofluorescence to locate deposited fibronectin. As seen in Fig 2, displacement of gold salts correlated well with deposited fibronectin as demonstrated by immunofluorescence, indicating that fibronectin tracks were deposited along the path traversed by the cell.

To further study the possibility that matrix proteins such as fibronectin may be involved in cellular movement, we plated keratinocytes on coverslips coated with gold salts and subsequently also treated with fibronectin. The coverslips were fixed in formalin and the cells viewed under dark-field microscopy. As shown in Fig 3, movement as indicated by displacement of particles was detectable on coverslips treated with fibronectin but not on controls and was more marked after longer incubations. Like spreading, movement was not affected by cycloheximide (25 μg/ml) for up to 8 h. The enhanced motility produced by fibronectin was concentration-dependent and was maximal at the concentrations required for maximal enhancement of spreading (not shown). In additional experiments comparing motility of keratinocytes on type I collagen and type IV collagen, these proteins were also found to enhance motility similarly (Fig 4). Enhanced motility on collagen was not mediated by fibronectin in the medium, since it was noted to occur both in medium A and in medium B, which lacked serum, and hence fibronectin.

As noted by Ali and Hynes [29] in several types of fibroblasts, nonmotile keratinocytes cleared a small area of gold particles from their periphery and left a clear ring. If cells were allowed to grow into colonies, a compact colony of nonmotile cells was surrounded by a clear ring. In the case of cells treated with fibronectin, however, extensive asymmetric areas were cleared of particles after several days before colonies developed. If cells were plated without gold particles, addition of fibronectin produced dispersed asymmetric cells similar to motile cells seen with gold particles and similar to motile cells described by Ali and Hynes with or without gold particles. Therefore, in our experiments with keratinocytes, as in those with fibroblasts, the gold particles did not appear to affect motility, and the effect of fibronectin appeared not to depend on the presence of the gold particles. The proteins in serum added with medium did not enhance motility, and addition of albumin (1 mg/ml), bovine pituitary extract (100 μg/ml), hydrocortisone (0.4 μg/ml), cholera toxin (5 ng/ml), or EGF (5 ng/ml), which also did not affect spreading, did not affect motility. Laminin did not alter motility (not shown). Cells plated on laminin-coated coverslips or with laminin in the medium (at 20 μg/ml) appeared comparable to control panels in Fig 3.

DISCUSSION

There is now substantial evidence that fibronectin may play a significant role in the biology of the keratinocyte. Examination of epidermis shows that fibronectin is found in the epidermis in a blister cavity [30] and in some pathologic conditions including basal cell carcinoma (reviewed in [31]), but not in normal epidermis. In vitro studies show secretion of soluble and insoluble fibronectin by human keratinocytes [12–15], enhanced growth on fibronectin of human keratinocytes [16],

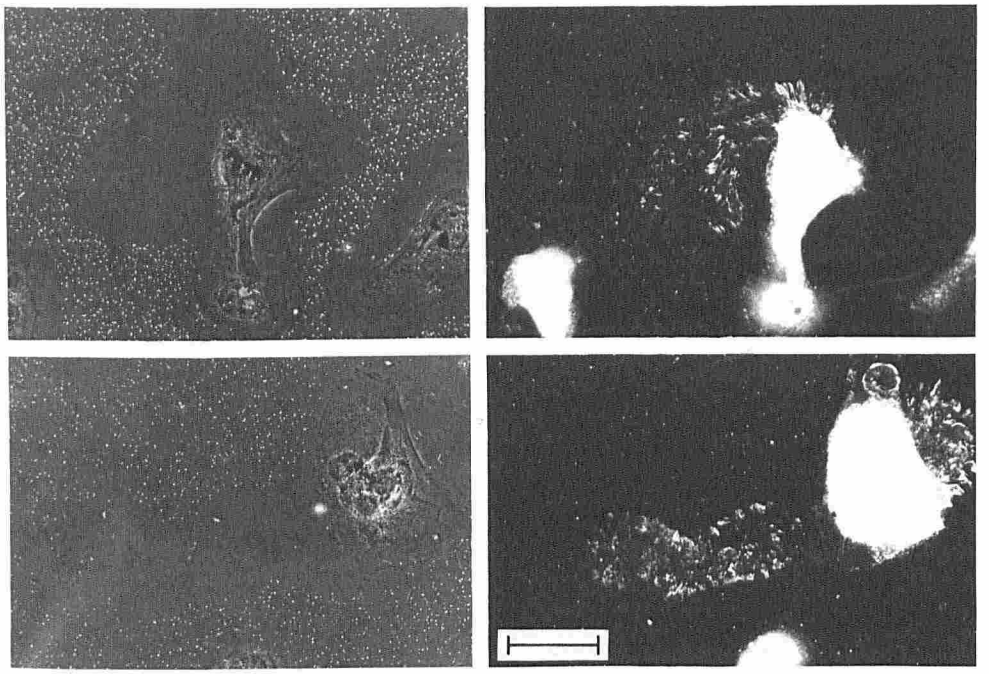

FIG 2. Relationship of phagokinetic tracks to insoluble fibronectin deposited by keratinocytes. Subcultured keratinocytes were plated on gold particle-coated coverslips in medium A and fixed after 2 days. Coverslips were processed for immunofluorescence with antifibronectin antibody and photographed under phase-contrast or fluorescence optics. *Left panels*, phase contrast; *right panels*, immunofluorescence. *Scale bar* = 150 μm.

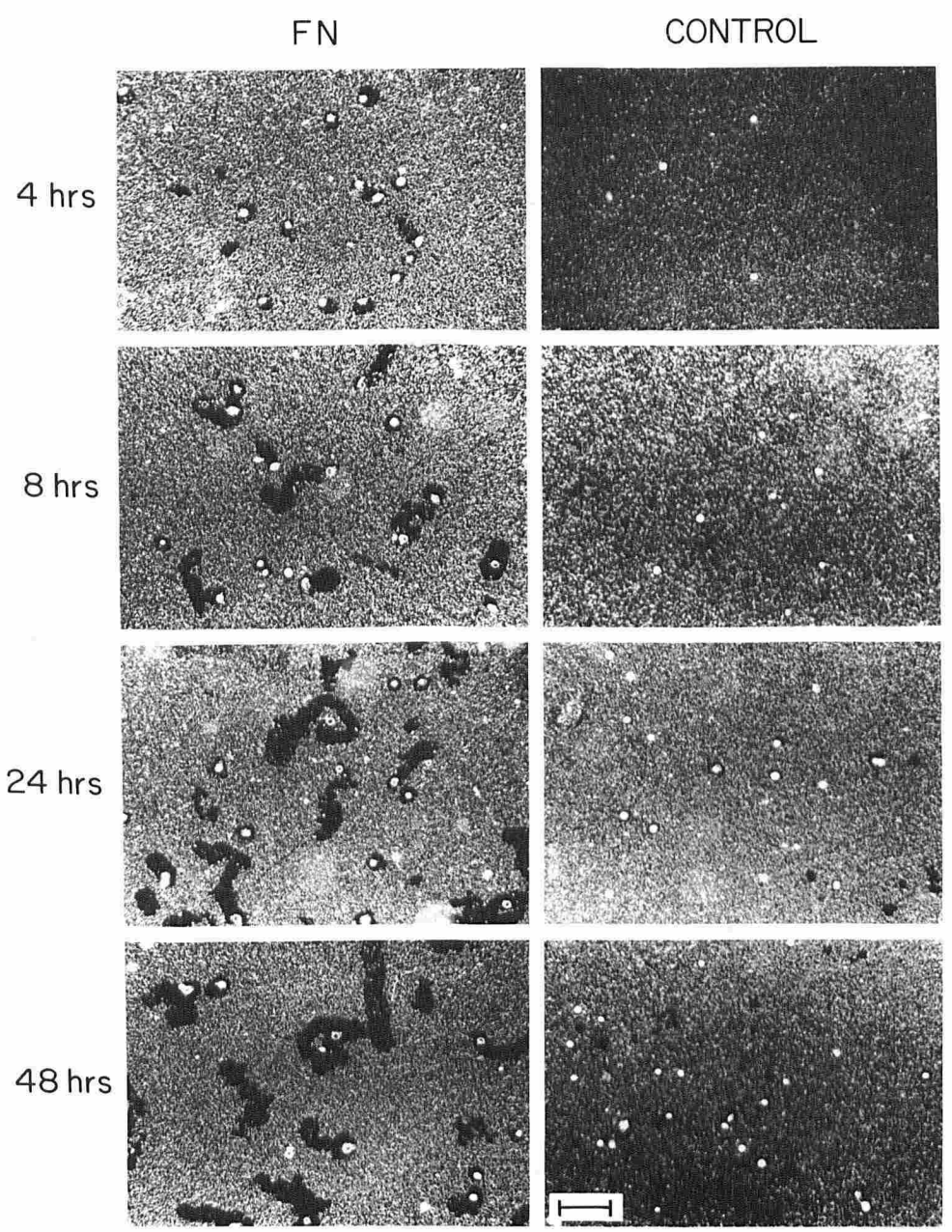

FIG 3. Enhanced motility of cultured keratinocytes on a fibronectin-coated surface. Coverslips with adherent gold particles were incubated with or without fibronectin (30 min, 37°C) in HBSS and then plated with cultured keratinocytes in medium B. Cells were fixed at various times and adherent cells photographed under dark-field optics. FN = Fibronectin. *Scale bar* = 50 μm.

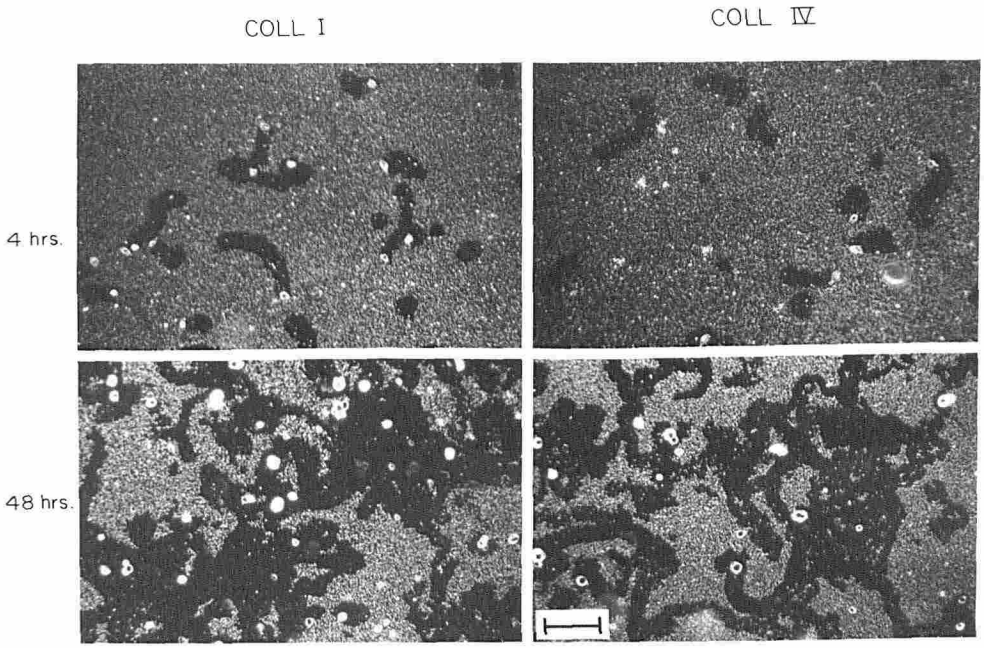

FIG 4. Enhanced motility of cultured keratinocytes on surfaces coated with type I (*COLL I*) or type IV (*COLL IV*) collagen. Coverslips with gold particles were incubated with or without collagen (30 min, 37°C) in HBSS and then plated with cultured keratinocytes in medium B and processed as in Fig 3. *Scale bar* = 50 μm.

and spreading on fibronectin of guinea pig keratinocytes [17]. We have now shown that fibronectin acts as a spreading factor for cultured human keratinocytes and that type IV collagen and type I collagen are also able to function in this way. Control cells incubated with serum- or albumin-containing medium did not spread. The ability of cells to spread on matrix factors does not require protein synthesis, since it is insensitive to cycloheximide. In the absence of a spreading factor, many attached cells will spread by 24 h (Table IV), although the percentage of spread cells is lower than the percentage which spreads rapidly on a preformed matrix of fibronectin or collagen. These cells presumably secrete or synthesize and secrete their own spreading factor or factors.

Not only is fibronectin able to speed apposition of the plasma membrane of attached cells to the substratum (spreading), but it is also associated with a more global type of movement, i.e., motility, in two important ways. First, since fibronectin deposits correlate with displacement of gold particles, keratinocytes leave behind a trail of insoluble fibronectin as they move across a culture surface (Fig 2). Second, a substratum of fibronectin, like type I or type IV collagen, is able to enhance motility of keratinocytes (Figs 3 and 4). Ali and Hynes [29] have demonstrated increased fibroblast motility on a fibronectin substratum. It is unclear whether keratinocytes require a substrate such as fibronectin to move on and therefore secrete it prior to movement, or whether it is left behind adventitiously and is not actually required for movement. It may be significant that keratinocyte fibronectin is found on the surface of the cell only between the cell and substratum [12], since this is probably the required location if fibronectin is to alter shape and motility of cells [17]. The fact that enhanced movement takes place in the presence of a previously laid down bed of fibronectin suggests that it or another matrix component is required for movement to occur. Although we have detected fibronectin tracks in cultures not treated with fibronectin [12], the number of cells making them is small in comparison with that of cells plated on fibronectin.

Since collagen can also increase motility, matrix proteins other than fibronectin may be laid down on these surfaces by motile cells. Stenn et al [32] reported that migrating epithelial sheets continually synthesized AB_2 (now called type V) collagen during migration, and studies with inhibitors suggested that such synthesis was required. Fibronectin, which interacts with collagen, was not sought in that study. Since Stenn et al used whole skin explants, the studies cannot be compared directly; it would be of interest, however, to examine keratinocytes for synthesis of type V collagen during stimulation of motility by fibronectin. Tchao has demonstrated enhanced motility of an epithelial cell line on collagen-coated surfaces [33] using time-lapse photography.

It has been suggested that since keratinocytes, unlike fibroblasts, do not utilize fibronectin for attachment to collagen [1], keratinocytes may have other specifically epithelial attachment factors, such as laminin [2,4]. Like fibroblasts [34], however, keratinocytes do synthestize and secrete soluble and insoluble fibronectin [12–15], they deposit fibronectin in paths of movement, and their spreading and motility are increased by added fibronectin. Furthermore, laminin may increase attachment of fibroblasts [35,36]. Fibronectin has recently been shown also to increase the spreading of an epithelial sheet from rabbit cornea in vitro [37]. This phenomenon may underlie the significance of fibronectin in the matrix of the healing wound as demonstrated by Grinnell et al [38] and by Clark et al [39]; fibronectin diffusing from serum may bind to collagen and to fibrin and serve as a substratum for the movement of epithelial cells which then cover a wound in vivo.

Additional evidence for interaction of keratinocytes with fibronectin was recently shown by Takashima and Grinnell, who demonstrated spreading of keratinocytes on fibronectin and phagocytosis of fibronectin-coated beads [40].

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