Fibronectin Receptors of Human Keratinocytes and Their Expression during Cell Culture

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Abstract. Keratinocyte attachment to fibronectin (FN) substrata was inhibited by the peptide Gly-*Arg-Gly-Asp-Ser*-Pro-Cys, but not by the variant peptide Gly-*Arg-Gly-Glu-Ser*-Pro. The RGDS-containing peptide did not inhibit keratinocyte adhesion to collagen. Keratinocyte adhesion to FN substrata also was inhibited by polyclonal anti-FN receptor antibodies originally prepared against the 140-kD FN receptors of Chinese hamster ovary (CHO) cells. Anti-CHO FN receptor antibodies did not, however, inhibit keratinocyte adhesion to collagen substrata. A monoclonal antibody designated VM-1 that was prepared against human basal keratinocytes inhibited keratinocyte adhesion to collagen but not to FN. Based on these results, we conclude that keratinocytes have distinct FN and collagen receptors. Experiments were performed to compare the expression of FN receptors on keratinocytes freshly isolated from skin and keratinocytes harvested from cell cultures. Cells harvested from keratinocyte cultures were able to neutralize the inhibitory activity of anti-CHO FN receptor antibodies and were able to attach and spread on anti-CHO FN receptor-coated substrata. Cells freshly harvested from skin, however, did not neutralize the antibodies, nor did they attach and spread on antibody-coated substrata. To learn more about the biochemical nature of the keratinocyte FN receptors, we performed immunoaffinity chromatography and immunoprecipitation experiments using the anti-CHO FN receptor antibodies. Extracts from metabolically radiolabeled, 10-d cultured keratinocytes contained FN receptors that had a 135-kD component under reducing conditions and 115- and 155-kD components under nonreducing conditions. Similar components were observed in extracts from surface-radiolabeled cells indicating that the FN receptors were expressed on keratinocyte cell surfaces. On the other hand, extracts from metabolically radiolabeled, 1-d cultured keratinocytes lacked intact FN receptors but contained a component that migrated at 48 kD under reducing conditions and 50 kD under nonreducing conditions. Because this fragment was not detected in surface-radiolabeled keratinocytes that were freshly isolated from skin, it seems likely that the fragment was located inside the cells rather than on the cell surface. A 50-kD FN receptor fragment also was observed in extracts from 10-d cultured keratinocytes if leupeptin and pepstatin were omitted from the extraction buffer. The results suggested that human keratinocytes cultured for 10 d express the 140-kD class of FN receptors, but that these receptors are not expressed on the surfaces of keratinocytes freshly isolated from skin. Probably, the 140-kD FN receptors of keratinocytes in skin are synthesized and then rapidly degraded cytoplasmically. The modulation of keratinocyte FN receptors is likely to be an important feature of reepithelization during wound repair.

BASAL keratinocytes in unwounded skin exist as nonmotile cells attached to a basement membrane that contains laminin and type IV collagen (17). During wound repair, however, these cells migrate over and through a fibronectin (FN)[1]-coated matrix (7, 13) in the absence of laminin and type IV collagen (27). Although it had been suggested that laminin was the adhesion ligand specific for epidermal cells (33), other studies did not support this idea (11, 28). Detailed examination of the adhesive properties of cultured human epidermal cells showed that FN was an adhesion ligand for these cells that promoted not only cell attachment and spreading, but also phagocytosis and motility (8, 20, 30, 31).

In the course of our experiments, we noticed that epidermal cells freshly isolated from human skin, unlike cultured cells, were unable to attach to FN substrata in short-term (45 min) assays (31). The onset of FN cell adhesion function was evident in epidermal cells harvested and tested after 2–4 d of culture and reached a maximal level after 10 d of culture. Epidermal cells activated during cell culture were found to be basal ketatinocytes based on indirect immunofluorescence staining with antikeratin antibodies and bullous pemphigoid

1. *Abbreviation used in this paper*: FN, fibronectin.

serum. A similar activation of FN cell adhesion function also occurred in keratinocytes that migrated out of epidermal explants (31).

Activation of FN cell adhesion function also was measured during wound healing in vivo (29). In these studies, rabbit ear epidermal cells were transplanted onto full-thickness wound beds that had been prepared on the backs of the same rabbits. It was found that keratinocytes harvested from grafts showed a markedly increased ability to attach and spread on FN substrata compared with keratinocytes freshly harvested from ear skin.

Studies performed to determine the specificity of epidermal cell attachment showed that cells freshly harvested from skin were able to attach to substrata other than FN including laminin/type IV collagen-containing basement membrane matrix produced by HR-9 cells and type I collagen (34). Moreover, unlike cell attachment to FN, there was little change in the ability of cells to attach to these other substrata after the cells were cultured. Therefore, the activation of cell attachment for FN that occurred during epidermal cell culture was specific.

The above findings led us to believe that keratinocyte FN receptors were activated during wound repair and under cell culture conditions. Although FN receptors of fibroblastic cells have been analyzed in detail (2, 4, 6, 15, 24, 32), little is known about the receptors of keratinocytes. Consequently, we became interested in learning more about human keratinocyte FN receptors. Toward this end, experiments were performed to test the effects of FN cell-binding domain peptides (1, 25) on keratinocyte adhesion. Also, we characterized keratinocyte FN receptors using a polyclonal anti-FN receptor antibody that was prepared originally against Chinese hamster ovary (CHO) FN receptors and found to cross-react with several human cell lines (4). The results of these studies are reported herein.

Materials and Methods

Cells

Human skin was obtained from the Burn Center, University of Texas Health Science Center, Dallas, and human epidermal cells were prepared using Dispase II (Boehringer Mannheim Biochemicals, Indianapolis, IN) and trypsin treatment and cultured as described previously (29–31). The culture medium consisted of Dulbecco's modified Eagle's medium (DME, Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS, Gibco), 20 mM Hepes buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 10 ng/ml epidermal growth factor (Sigma Chemical Co., St. Louis, MO), 10^{-9} M cholera toxin (Sigma Chemical Co.), and 0.4 μg/ml hydrocortisone (Sigma Chemical Co.). For 5- or 10-d (confluent) cultures, cells freshly harvested from skin (1×10^7) in 15 ml of culture medium were plated on 75-cm^2 tissue culture flasks (Falcon Labware, Oxnard, CA) and incubated at 37°C in a humidified incubator with 5% CO_2. The first medium change was 2 d after initiating the cultures. Throughout the culture period, the medium was changed two to three times per week.

Some experiments were performed with cells that were freshly harvested from skin or cultured for only 1 d. In this case, it was desirable to obtain a preparation enriched in basal keratinocytes, which account for only ~30% of the keratinocytes in skin but account for ~80% of keratinocytes in the 5- or 10-d cultures (34). To obtain enriched preparation, freshly harvested cells (2×10^7) in 15 ml of culture medium were plated for 45 min on 75-cm^2 flasks previously coated with 50 μg/ml collagen as described previously (34).

Cells cultured on plastic or on collagen-coated plastic were harvested using the same combination of Dispase and trypsin treatment used to obtain cells from human skin. Prior to experiments, harvested cells were allowed to recover from enzyme treatments by incubation for 2 h at 37°C in culture medium. Epidermal cell preparations used in the experiments described in this paper were ~90% keratinocytes based on staining with antikeratin antibodies and 80% basal cells based on staining with bullous pemphigoid serum (31, 34).

Materials and Preparation of Substrata

FN was obtained from the New York Blood Center. FN-coated substrata were prepared as described previously (29–31) by incubating 35-mm tissue culture dishes (Falcon Labware) with 20 μg/ml FN in phosphate-buffered saline (PBS) (10 mM Na phosphate, 150 mM NaCl, pH 7.2) and countercoating the dishes with 1% heat-denatured bovine serum albumin (BSA) (crystallized, Miles Scientific, Naperville, IL).

Collagen samples (3 mg/ml) (Vitrogen 100, Collagen Corp., Palo Alto, CA) were brought to physiological ionic strength with 10× PBS and neutralized with NaOH. Collagen-coated substrata were prepared as before (34) by incubating tissue culture dishes (above) for 30 min at 37°C with 3 ml of DME containing 50 μg/ml neutralized collagen and rinsing with DME.

Polyclonal antibodies to 140-kD receptors of CHO cells were isolated as described previously (4). The IgG fraction obtained by ammonium sulfate fractionation was passed through a gelatin-sepharose column to remove FN contaminating the preparation (10), and the absence of FN was confirmed by SDS-PAGE (data not shown). Antibody-coated substrata were prepared by incubating tissue culture dishes for 10 min at 22°C with 2.4 mg/ml antibody in PBS followed by 1% heat-denatured BSA.

Monoclonal VM-1 antibody hybridoma supernatant (22) was a generous gift from Dr. Vera Morhenn (Stanford Medical Center, Stanford, CA). Synthetic peptides Gly-Arg-Gly-Asp-Ser-Pro-Cys (GRGDSPC) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) were purchased from Peninsula Laboratories, Inc., Belmont, CA.

Cell Attachment and Spreading Assays

Keratinocyte attachment and spreading were measured as before (29–31). Freshly harvested cells or harvested cultured cells (2×10^5) in 2 ml of DME were incubated on the test substrata for 45 min at 37°C in a humidified incubator with 5% CO_2. At the end of the incubations, the dishes were subjected to shaking at 150 rpm for 10 s on a reciprocating shaker (model R-2, New Brunswick Scientific Co., Inc., Edison, NJ), and nonattached cells were removed with a pipette. Cell attachment was quantified visually by counting 20 random 0.6-mm^2 microscopic fields and calculating the number of cells attached per dish. The percentage of attached cells that were spread (with obvious cytoplasmic reorganization around the nucleus) was determined visually by counting 100–150 cells. Typically, maximal attachment of keratinocytes in these short-term assays is 50–60%, and 80% of the cells that attach are basal keratinocytes (34).

Protein Determination

Protein concentration was determined by the fluorescamine method using the fluorescamine assay reagent according to the manufacturer's instructions (Roche Diagnostics, Nutley, NJ). BSA was used as the standard.

Immunoabsorption

Cells freshly harvested from skin or harvested from cell cultures (and allowed to recover for 2 h in growth medium) were fixed for 20 min at 22°C with 3% formaldehyde in PBS, washed twice with PBS, treated for 10 min at 22°C with PBS containing 1% BSA and 1% glycine, and washed twice again with PBS. Cells were resuspended in 0.1 ml of DME and incubated for 60 min at 22°C with 2 mg of anti-CHO FN receptor IgG or nonimmune goat IgG. At the end of incubations, the samples were microfuged to remove the cells, and the supernatants were tested for their effects on epidermal cell adhesion to FN substrata.

Immunoaffinity Chromatography

Metabolically radiolabeled epidermal cells were obtained by incubating cell cultures for 16 h with 8 ml of methionine-deficient medium containing 20 μCi/ml [^{35}S]methionine. Each 75-cm^2 flask of cells was extracted with 3 ml of cold NP-40 extraction buffer (137 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 1% NP-40, 10% glycerol, 1 mM EDTA, 2 mM Phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, pH 8.0). After 1 min, cells were scraped from the flasks, and the flasks were washed with an additional 3 ml of extraction buffer. The pooled samples (usually 10 flasks per experiment, ~9×10^7 radiolabeled cells) were incubated for 30 min at 4°C in extraction buffer after which insoluble

material was removed by centrifugation for 1 h at 37,000 *g* (Sorvall Ti 50.2 rotor, DuPont-Sorvall, Newtown, CT).

Molecules bound by polyclonal anti-CHO FN receptor antibodies were isolated by affinity chromatography. Cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) was coupled with immune IgG or nonimmune goat IgG according to the manufacturer's instructions (10 mg IgG/g gel). Before use, columns were washed with 15 ml of acetate buffer (0.1 M Na acetate, 0.5 M NaCl, pH 4.0) and 15 ml of running buffer (0.1 M Tris, 0.5 M NaCl, pH 8.0). The NP-40 extracts of radiolabeled epidermal cells were chromatographed sequentially through 10 ml of Sepharose 2B (Pharmacia Fine Chemicals), 10 ml of nonimmune IgG-coupled Sepharose 4B, and 2 ml of immune IgG-coupled Sepharose 4B. The columns were washed with 200 ml of running buffer and eluted with 20 ml of 0.1 M glycine-HCl (pH 2.5). The eluted fractions were immediately titrated to pH 7-7.5 with Tris base and dialyzed exhaustively against 0.005 M Tris HCl, pH 7.4, and lyophilized.

Immunoprecipitation

Immunoprecipitation of ^{125}I surface-radiolabeled keratinocytes with anti-CHO FN receptor antibodies was performed as has been described (4). Briefly, rabbit anti-goat IgG (Cooper Biomedical, Inc., Malvern, PA)-conjugated protein A Sepharose 4B beads (Pharmacia Fine Chemicals) were preincubated with nonimmune IgG or anti-CHO FN receptor antibodies. Freshly isolated keratinocytes enriched for basal cells or keratinocytes harvested from 10-d cultures (2×10^7) were surface radiolabeled with 1 mCi Na[^{125}I] using Iodo-Beads (Pierce Chemical Co., Rockford, IL) according to the manufacturer's directions and then extracted with NP-40 extraction buffer (see above) for 30 min at 4°C. The extracts were centrifuged at 12,000 *g* in a microfuge (model 11, Beckman Instruments, Inc., Fullerton, CA) for 15 min. Cell extracts first were incubated for 2 h at 4°C with nonimmune goat IgG-beads and then incubated overnight at 4°C with anti-CHO FN receptor beads. Finally, the beads were washed three times with NP-40 extraction buffer, once with PBS containing 0.5 M NaCl, and one more time with NP-40 extraction buffer. The immunoprecipitated materials associated with beads after the washing procedures were solubilized in sample buffer for electrophoresis and autoradiography.

Electrophoresis and Fluorography

Samples for electrophoresis were boiled for 3 min in nonreducing sample buffer (62.5 mM Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue, pH 6.8) or reducing sample buffer (same as above plus 5% mercaptoethanol). Electrophoresis was performed as previously described using 4–16% acrylamide gradient slab gels containing a gradient of 3–8 M urea (16, 21). Gels for autoradiography and fluorography were prepared using AcrylAide (FMC Corp., Rockland, ME) as the cross-linker instead of *N-N'*-methylene-*bis*-acrylamide and supported with GelBond-PAG film (FMC Corp.). After electrophoresis, gels were fixed and stained with Coomassie Blue. Gels to be fluorographed were impregnated with En^{3}Hance (containing 2% glycerol) for 2 h at 22°C. Gels were dried in a 60°C oven and exposed to Kodak XAR-5 film on a Cronex intensifying screen (Dupont Co., Wilmington, DE).

Results

Effect of Peptides on Keratinocyte Adhesion

The first series of experiments carried out were designed to learn whether keratinocytes attached to the same domain of FN that has been identified in attachment of fibroblasts. To accomplish this, we tested the effect of an Arg-Gly-Asp-Ser peptide on keratinocyte adhesion. Addition of GRGDSPC at concentrations ranging from 0.0625 to 1.0 mg/ml resulted in inhibition of both keratinocyte attachment and spreading on FN substrata (Fig. 1). The concentration required for half-maximal inhibition was between 0.125 and 0.250 mg/ml. In marked contrast, there was no inhibition of keratinocyte attachment or spreading on collagen substrata at any of the peptide concentrations tested (Fig. 1). Also, the substituted peptide GRGESP did not inhibit cell adhesion on FN or collagen substrata (data not shown).

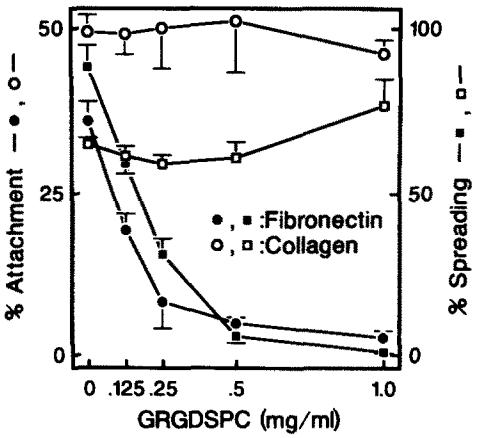

Figure 1. Effect of synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro-Cys (GRGDSPC) on keratinocyte adhesion. Keratinocytes harvested from confluent cell cultures were tested for cell attachment and spreading on FN and collagen substrata in the presence of GRGDSPC at the concentrations indicated. The data shown are the averages ± SD from three separate experiments. Other details are in Materials and Methods.

Effect of Anti-CHO FN Receptor and VM-1 Antibodies on Keratinocyte Adhesion

The above results showed that human keratinocytes interacted with the RGDS-containing cell binding domain of FN. Also, we tested the effect of polyclonal anti-CHO FN receptor antibodies on keratinocyte adhesion. This antibody inhibited the attachment and spreading of human keratinocytes to FN substrata but not to collagen substrata (Fig. 2). Similar concentrations of nonimmune goat IgG had no effect on adhesion (data not shown). Conversely, the monoclonal antibody VM-1 (22) inhibited human keratinocyte attachment to collagen but not to FN (Fig. 3). These results show that human keratinocyte adhesion to different substrata can be selectively inhibited with different antibodies.

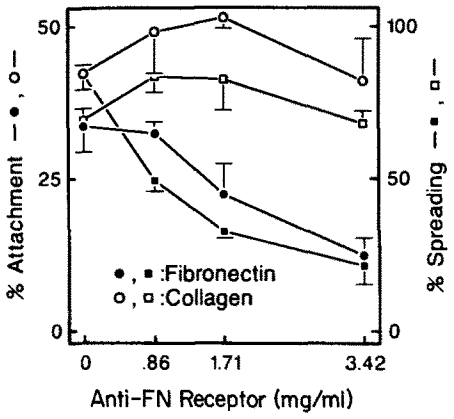

Figure 2. Effect of anti-CHO FN receptor antibodies on keratinocyte adhesion to FN and collagen substrata. Keratinocytes harvested from confluent cell cultures were tested for cell attachment and spreading on FN and collagen substrata in the presence of anti-CHO FN receptor antibodies at the concentrations indicated. Data shown are the averages ± SD from three separate experiments. Other details are in Materials and Methods.

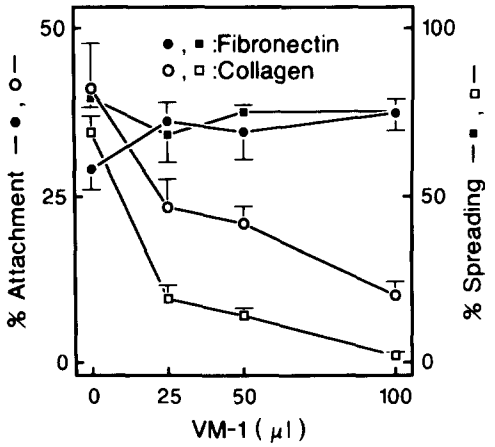

Figure 3. Effects of VM-1 antibodies on keratinocyte adhesion to FN substrata. Keratinocytes harvested from confluent cell cultures were tested for cell attachment and spreading on FN and collagen substrata in the presence of VM-1 antibodies at the concentrations indicated. Data shown are the averages ± SD from two separate experiments. Other details are in Materials and Methods.

Expression of FN Receptors on Keratinocytes Freshly Harvested from Skin Compared with Cultured Keratinocytes

The foregoing results suggested that keratinocytes possessed the 140-kD class of FN receptors because keratinocyte adhesion to FN was inhibited by anti-CHO FN receptor antibodies. To compare the expression of these FN receptor binding sites on the surfaces of keratinocytes freshly harvested from skin or harvested from cultures, quantitative immunoabsorption-neutralization experiments were performed. Keratinocytes freshly harvested from skin or harvested from confluent cell cultures (by the same combination of Dispase and trypsin treatment) were fixed, and various cell numbers were incubated with anti-CHO FN receptor antibodies. Subsequently, the cells were removed and the remaining supernatants were tested for their abilities to inhibit adhesion of cultured keratinocytes to FN (Fig. 4).

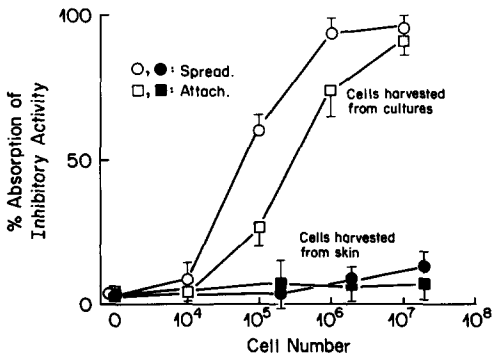

Figure 4. Absorption of anti-CHO FN receptor antibody with freshly isolated and cultured keratinocytes. Samples of anti-CHO FN receptor antibodies were incubated with fixed keratinocytes that were freshly harvested from skin or harvested from cell cultures as indicated. After removing the cells, the ability of the remaining antibody preparation to inhibit keratinocyte adhesion to FN substrata was tested. Data shown are the averages ± SD from two separate experiments. Other details are in Materials and Methods.

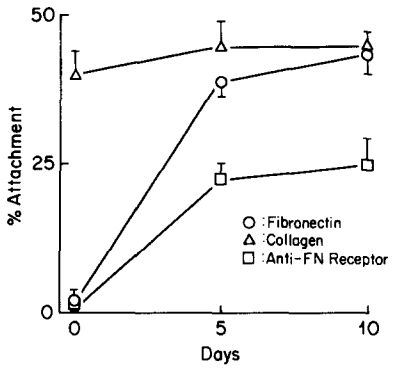

Figure 5. Attachment of freshly isolated and cultured keratinocytes on FN, collagen, and anti-CHO FN receptor substrata. Keratinocytes, freshly harvested from skin or harvested from 5- or 10-d cell cultures, were tested for cell attachment on the substrata indicated. Data shown are the averages ± SD from two separate experiments. Other details are in Materials and Methods.

In the absence of cells or with 10^4 cells, there was no absorption of inhibitory activity. Above 10^5 cells, the cultured keratinocytes absorbed inhibitory activity from the anti-CHO FN receptor preparation, and the inhibitory activity was neutralized completely with 10^7 cells. On the other hand, cells freshly harvested from skin do not absorb inhibitory activity from the anti-CHO FN receptor preparation. This result suggested that FN receptors were expressed on the surfaces of cultured keratinocytes but not on the surfaces of keratinocytes freshly harvested from skin.

Keratinocyte attachment and spreading assays on substrata coated with anti-CHO FN receptor IgG also were performed. Cells freshly isolated from skin were unable to attach to fibronectin or anti-CHO FN receptor antibody substrata, but these cells were able to attach to collagen substrata (Fig. 5). Cells harvested from 5- or 10-d-old cultures, however, showed markedly increased attachment to fibronectin and anti-CHO FN receptor substrata although the extent of cell attachment to collagen substrata did not change. Increased cell spreading on anti-CHO FN receptor antibodies was observed over the same time that cell spreading on FN and collagen substrata increased (Fig. 6). These results are

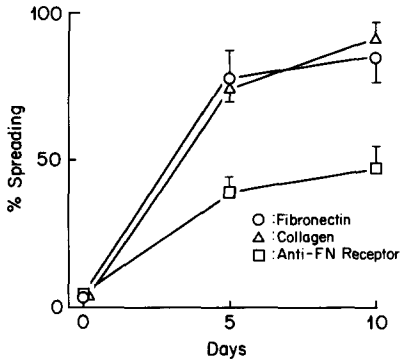

Figure 6. Spreading of freshly isolated and cultured keratinocytes on FN, collagen, and anti-CHO FN receptor-coated substrata. Keratinocytes, freshly harvested from skin or harvested from 5- or 10-d cell cultures, were tested for cell spreading on the substrata indicated. Data shown are the averages ± SD from two separate experiments. Other details are in Materials and Methods.

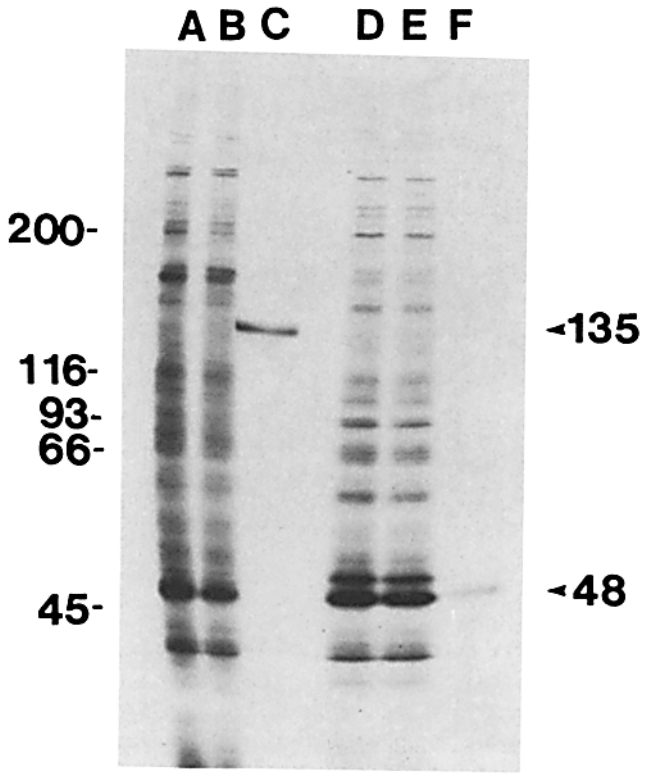

Figure 7. Fibronectin receptors from metabolically radiolabeled keratinocytes analyzed by SDS-PAGE under reducing conditions. Metabolically radiolabeled keratinocytes were harvested from 10-d (*A–C*) or 1-d (*D–F*) cultures. Cells were extracted with NP-40 (*A*, *D*). Most of the radiolabeled cell components passed through an anti-CHO FN receptor antibody column (*B*, *E*). With the extracts of cells harvested from 10-d cultures, a 135-kD component bound to the column and was eluted by glycine-HCl (*C*). With the extracts from the 1-d cultures, a 48-kD component bound the column (*F*). Specimens for electrophoresis were prepared in reducing sample buffer. 10,000 cpm were loaded on each lane. Other details are in Materials and Methods. M_r standards given in kilodaltons.

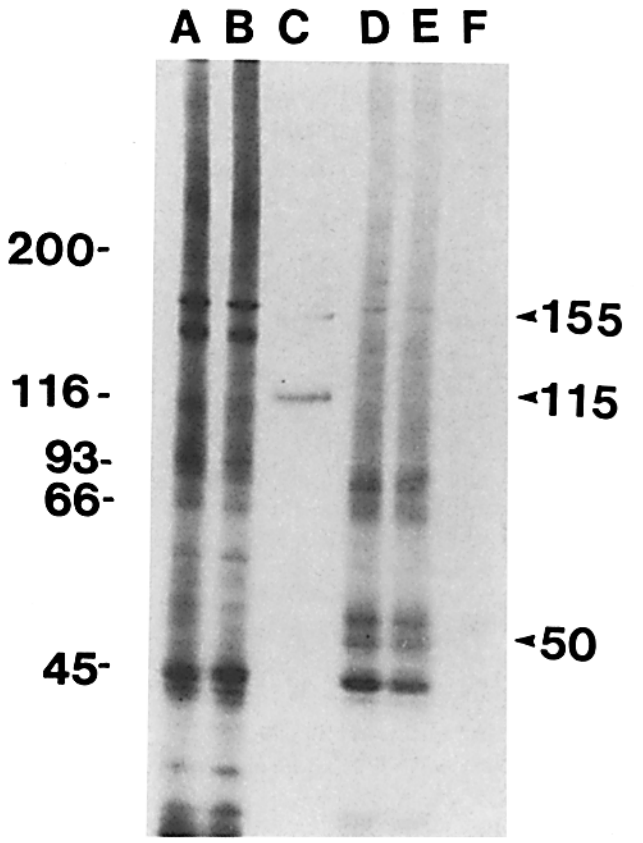

Figure 8. Fibronectin receptors from metabolically radiolabeled keratinocytes analyzed by SDS-PAGE under nonreducing conditions. Same as Fig. 7 except specimens for electrophoresis were prepared in nonreducing sample buffer. With the extracts of cells from 10-d cultures, 115- and 155-kD components bound to the column and were eluted by glycine-HCl (*C*). With the extracts from the 1-d cultures, a 50-kD component bound the column (*F*). Other details are in Materials and Methods. M_r standards given in kilodaltons.

consistent with the antibody neutralization studies (above) and suggest that FN receptors are expressed on cultured keratinocytes but not on keratinocytes freshly harvested from skin.

Immunoaffinity Chromatography of Human Keratinocyte FN Receptors

To learn more about the biochemical nature of keratinocyte FN receptors, cell extracts were analyzed by immunoaffinity chromatography. In these experiments, we used cells from 10-d cultures and cells from 1-d cultures that have no detectable FN receptor function (31, 34). Metabolically radiolabeled keratinocytes were extracted with NP-40 buffer and chromatographed sequentially on native Sepharose, nonimmune IgG-coupled Sepharose, and anti-CHO FN receptor IgG-coupled Sepharose columns. Most of the starting cell extract protein passed through the columns. After washing, the columns were eluted with 0.1 M glycine-HCl, and no radioactive components could be recovered from the native Sepharose or nonimmune columns. There was, however, a small but reproducible amount of material representing <0.02% of the starting preparation that was eluted from the immune column.

Analysis of column fractions by SDS-gel electrophoresis under reducing conditions showed a large number of components ranging from <45 kD to >300 kD. The profile of these components was similar although not identical in the extracts from keratinocytes cultured for 10 d (Fig. 7, lane *A*) compared with extracts from cells that were cultured 1 d (Fig. 7, lane *D*). Also, the profiles of unbound material that passed through the immune column were essentially identical to the starting cell extracts (Fig. 7, lane *B* vs. *A* and *E* vs. *D*). In the extract from cells cultured 10 d, there was a single component of 135 kD that bound to and was eluted from the immune column (Fig. 7, lane *C*). In marked contrast, the extract from cells cultured 1 d did not contain the 135-kD molecule. Rather, they contained a lower-molecular-mass component of 48 kD that was eluted from the immune column (Fig. 7, lane *F*). Specimens also were analyzed by SDS-gel electrophoresis under nonreducing conditions. In this case, the 135-kD component from 10-d cultured keratinocytes was seen to migrate as two components of 115 and 155 kD (Fig. 8, lane *C*). The lower-molecular-mass component

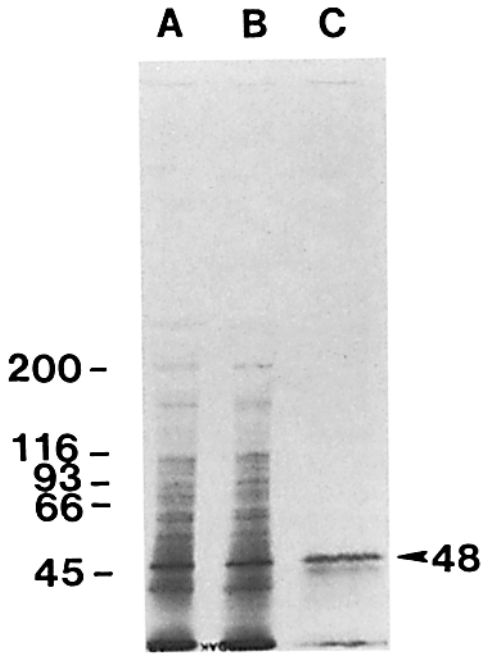

Figure 9. Partially degraded fibronectin receptors from keratinocytes cultured 10 d. Same as Fig. 7 (lanes *A–C*) except pepstatin and leupeptin were omitted from the NP-40 extraction buffer. High-molecular-mass components were absent from the cell extracts (*A*) and the fraction that did not bind to the affinity column (*B*). A 48-kD component bound to the affinity column and was eluted by glycine-HCl (*C*). 50,000 cpm were loaded on lanes *A* and *B*; 5,000 cpm were loaded on lane *C*. Other details are in Materials and Methods. M_r standards given in kilodaltons.

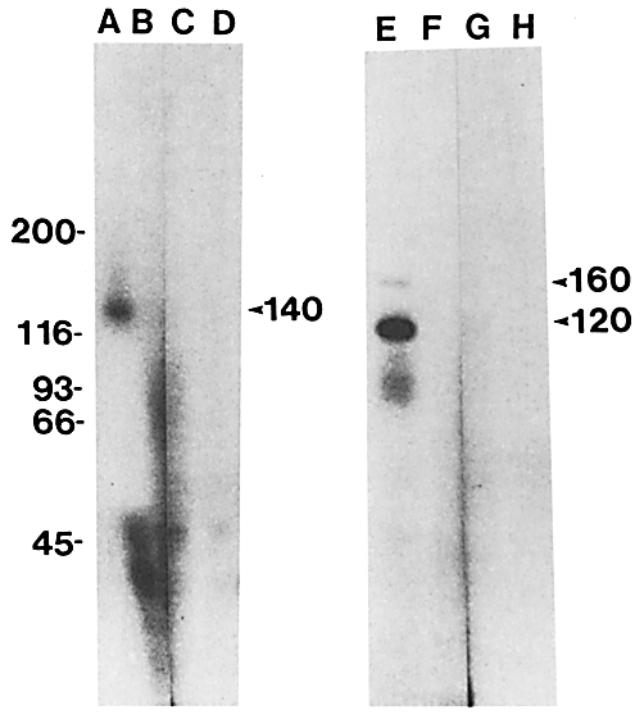

Figure 10. Fibronectin receptors from radioiodinated keratinocytes analyzed by SDS-PAGE. Radioiodinated keratinocytes were prepared with cells harvested from 10-d cultures (*A, B, E, F*) or cells freshly isolated from skin and enriched in basal keratinocytes (*C, D, G, H*). Radioiodinated cells were extracted with NP-40 and immunoprecipitated sequentially with nonimmune goat IgG beads (*B, D, F, H*) followed by anti-CHO FN receptor antibody beads (*A, C, E, G*). From the extracts of cells harvested from 10-d cultures, components were specifically immunoprecipitated that migrated with a molecular mass of 140 kD under reducing conditions (*A*) and 120 and 160 kD under nonreducing conditions (*E*). No specific components were detected by immunoprecipitation of the extracts of cells freshly isolated from skin (*C, G*). Specimens for electrophoresis were prepared in reducing sample buffer (*A–D*) or nonreducing sample buffer (*E–H*). 5,000 cpm were loaded on each lane. Other details are in Materials and Methods. M_r standards given in kilodaltons.

from 1-d cultured keratinocytes was present and had a molecular mass of 50 kD (Fig. 8, lane *F*). These results indicate that 10-d cultured human keratinocytes have FN receptors similar to those found in mammalian fibroblasts (4, 24). On the other hand, 1-d cultured keratinocytes, which have been shown to lack normal FN receptor function, appeared to lack intact FN receptors.

While working out optimal extraction conditions with cultured keratinocytes, we found that the cell extracts had a high level of endogenous protease activity. Consequently, if we added only PMSF to the extraction buffer instead of PMSF, leupeptin, and pepstatin, then the high-molecular-mass components in the keratinocyte extract were lost (Fig. 9, lane *A* compared with Fig. 7, lane *A*), and the predominant component that was eluted from the immune column was ~50 kD (Fig. 9, lane *C*). This finding indicated that the 48-kD component found in the extracts from 1-d cultures (Fig. 7, lane *F*) might be a proteolytic fragment of 140-kD FN receptors.

Immunoprecipitation of Human Keratinocyte FN Receptors

To confirm that the keratinocyte FN receptors detected in studies with metabolically radiolabeled cells were expressed at the cell surface, immunoprecipitation studies were performed with surface-radiolabeled keratinocytes. In addition to cells from 10-d cultures, cells freshly isolated from skin and enriched for basal cells were tested. The keratinocytes were radioiodinated, extracted with NP-40 buffer, and immunoprecipitated sequentially with nonimmune IgG-beads and anti-CHO FN receptor antibody beads.

Keratinoctyes from radioiodinated 10-d cell cultures had cell surface components that were specifically immunoprecipitated by anti-CHO FN receptor antibody beads. These components had molecular masses of 140 kD under reducing conditions (Fig. 10, lane *A*) and 120 and 160 kD under nonreducing conditions (Fig. 10, lane *E*), which indicated that the FN receptors detected in metabolically radiolabeled cells were expressed on the cell surface. On the other hand, the lower-molecuar-mass FN receptor fragment observed in the extracts of metabolically radiolabeled, 1-d cultured keratinocytes was not detected by immunoprecipitation of surface-labeled, freshly isolated keratinocytes. It is possible, therefore, that FN receptors are synthesized in freshly isolated keratinocytes, but degraded before they reach the cell surface.

Discussion

The purpose of our studies was to analyze FN receptors of human keratinocytes. We found that the FN receptors of these cells recognized the RGDS-containing, cell-binding do-

main of FN. That is, attachment to FN substrata was inhibited by the peptide Gly-*Arg-Gly-Asp-Ser*-Pro-Cys, but not by the variant peptide Gly-*Arg-Gly-Glu-Ser*-Pro. Similar findings have been made for a variety of other cell types including fibroblasts, platelets, and macrophages (1, 25). Interestingly, the RGDS-containing peptide did not inhibit keratinocyte adhesion to collagen, and others recently reported that a very similar peptide was a poor inhibitor of human osteosarcoma adhesion to collagen (9). These results are consistent with the idea that cell adhesion to native collagen is independent of fibronectin, which we suggested for fibroblasts some time ago (14) and was subsequently confirmed by others (26). This idea also is supported by the result that keratinocytes freshly harvested from skin are able to attach to collagen substrata but not to FN substrata (34).

Keratinocyte adhesion to FN substrata also was inhibited by a polyclonal anti-CHO FN receptor antibody. Previously (4), the anti-CHO FN receptor antibody was shown to be related to antibodies recognizing the 110–160-kD adhesion protein complex of chick fibroblasts (6, 15) and the 140-kD FN receptor of human osteosarcoma cells (24). In our experiments, we found that the anti-CHO FN receptor antibody did not inhibit keratinocyte adhesion to collagen substrata, which is further evidence that this antibody is very selective for FN-mediated cell adhesion (3, 4). On the other hand, the monoclonal antibody VM-1 inhibited cell attachment to collagen but not to FN substrata, further supporting a role for different cell receptors in adhesion to FN and collagen substrata.

Both the antibody neutralization experiments and keratinocyte attachment and spreading experiments on substrata coated with anti-CHO FN receptor antibodies led to the conclusion that FN receptors are expressed on cultured keratinocytes but not on keratinocytes in unwounded skin. This difference was unlikely to be accounted for by differences in the methods for preparing the cells because the enzyme treatments used to harvest cells were the same for skin and for cell cultures, and the harvested cells were allowed to recover in medium for 2 h before they were tested.

The biochemical nature of keratinocyte FN receptors was analyzed by immunoaffinity chromatography and immunoprecipitation experiments using the anti-CHO FN receptor antibodies. Extracts from metabolically radiolabeled, 10-d cultured keratinocytes contained typical mammalian FN receptors (4, 24) that had a 135-kD component under reducing conditions and 115- and 155-kD components under nonreducing conditions. Similar components were observed in extracts from surface-radiolabeled cells indicating that the FN receptors were expressed on keratinocyte cell surfaces. On the other hand, extracts from metabolically radiolabeled, 1-d cultured keratinocytes lacked intact FN receptors but contained a component that migrated in SDS-PAGE at 48 kD under reducing conditions and 50 kD under nonreducing conditions. A similar component also was observed in extracts from 10-d cultured keratinocytes if leupeptin and pepstatin were omitted from the extraction buffer. This intriguing observation suggested that 1-d cultured keratinocytes contain a proteolytic fragment of FN receptors. Because this fragment was not detected in surface-radiolabeled keratinocytes that were freshly isolated from skin, it seems likely that the fragment was located inside the cells rather than on the cell surface.

The above results suggest a molecular explanation for the functional studies described in this paper and previously. That is, human keratinocytes cultured for 10 d express the 140-kD class of FN receptors, but these receptors are not expressed on the surfaces of keratinocytes freshly isolated from skin. Probably, the 140-kD FN receptors of keratinocytes in skin are synthesized and then degraded cytoplasmically. Modulation of FN receptor expression in cultured keratinocytes is consistent with the idea that these receptors are activated during wound repair as discussed at the beginning of this article. This activation may be an essential aspect of wound repair in that a FN matrix has been implicated in epidermal migration as was discussed elsewhere (12). In this regard, it is worthwhile pointing out that topical application has been reported to promote epithelialization of nonhealing corneal ulcers (18, 19) and nonhealing venous stasis ulcers (35).

Finally, it should be noted that developmental changes in the expression of FN receptors also have been reported. In murine erythroleukemia cells, for instance, it was found that the 140-kD FN receptors disappeared after dimethyl sulfoxide-induced differentiation of these cells (23). Consistent with this finding, reticulocytes but not erythrocytes from peripheral blood express the 140-kD receptors. Thus, loss of the receptors may be a feature of terminal differentiation during hematopoiesis. In another study, it was found that the 140-kD class of FN receptors was present on embryonic chick lung cells, but markedly reduced in differentiated cells except smooth muscle (5). Future studies will be necessary to clarify the molecular mechanisms that control developmental changes in FN receptor expression and the modulation of FN receptor expression during wound repair.

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