

# Hypoxia Increases Human Keratinocyte Motility on Connective Tissue

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## Abstract

Re-epithelialization of skin wounds depends upon the migration of keratinocytes from the cut margins of the wound and is enhanced when human keratinocytes are covered with occlusive dressings that induce hypoxia. In this study, two independent migration assays were used to compare cellular motility on connective tissue components under normoxic or hypoxic conditions. Human keratinocytes apposed to collagens or fibronectin exhibited increased motility when subjected to hypoxic (0.2 or 2% oxygen) conditions compared with normoxic (9 or 20% oxygen) conditions. When compared with normoxic cells, hypoxic keratinocytes exhibited increased expression and redistribution of the lamellipodia-associated proteins (ezrin, radixin, and moesin). Furthermore, hypoxic keratinocytes demonstrated decreased secretion of laminin-5, a laminin isoform known to inhibit keratinocyte motility. Hypoxia did not alter the number of integrin receptors on the cell surface, but did induce enhanced secretion of the 92-kD type IV collagenase. These data demonstrate that hypoxia promotes human keratinocyte motility on connective tissue. Hypoxia-driven motility is associated with increased expression of lamellipodia proteins, increased expression of collagenase and decreased expression of laminin-5, the locomotion brake for keratinocytes. (*J. Clin. Invest.* 1997; 100: 2881–2891.) **Key words:** re-epithelialization • integrins • laminin-5 • collagenase • cytoskeleton

## Introduction

Conventional wisdom is that the provision of significant oxygen is necessary for proper healing of skin wounds (1, 2). Elderly individuals with ischemic vascular disease and poor perfusion to their lower extremities are frequently plagued by skin ulceration and poor healing. Tissue oxygen measurements have been used to study the effect of oxygen on healing wounds. Noninvasive measurements using transcutaneous oximetry and Laser doppler flowmetry clearly indicate that the degree

of tissue ischemia correlates with poor healing (3–5). Hyperbaric oxygen chambers have been built at great expense and used for the treatment of nonhealing wounds (6). Nevertheless, sometimes venous ulcers heal even when the transcutaneous oxygen pressure is markedly reduced (7).

One major advancement in the healing of cutaneous wounds is the use of semioclusive dressings (hydrogels, hydrocolloids, and polyurethane membranes). These dressings promote re-epithelialization and wound closure compared with wounds allowed to air-dry (8–12), but must be used early after wounding to induce rapid re-epithelialization in vivo (13). Wounds allowed to air-dry have oxygen tensions between 1–4% at the surface where keratinocytes are migrating (4, 14). In contrast, under occlusive dressings, the pH is very low and the oxygen tension is almost negligible (15). Yet, these dressings clearly promote re-epithelialization compared with wounds allowed to air-dry that have a higher oxygen tension at the wound surface.

Wound healing is a complex phenomenon that has several distinct components including coagulation, re-epithelialization, angiogenesis, fibroplasia, contraction, and remodeling (16). It is conceivable that each of these processes may have distinct responses to microenvironmental factors such as hypoxia. Re-epithelialization is the process by which the keratinocytes migrate horizontally from the wound margins across the wound bed and resurface the wound with an epithelium. Keratinocyte motility is an early event in the process of re-epithelialization (16). We have had experience with methods that directly measure keratinocyte motility in response to extracellular matrix and growth factors (16–18). In this study, we examined the effects of hypoxia on human keratinocyte migration over connective tissue components.

## Methods

**Keratinocyte cultures.** Human keratinocytes obtained from surgical skin specimens (two adults, ages 34 and 46) and neonatal foreskins (10 different donors) were cultured in low calcium, serum-free medium (SFM; GIBCO BRL, Gaithersburg, MD) or Keratinocyte Growth Medium (KGM;<sup>1</sup> Clonetics, San Diego, CA) as described by Boyce and Ham (19) and modified by O'Keefe and Chiu (20).

**Oxygen systems.** The standard oxygen level was defined as the pO<sub>2</sub> which exists in a standard, conventional, humidified tissue culture incubator at 37°C (20%). The low (0.2–2%) and tissue normoxia (9%) oxygen systems were established in a humidified environmental chamber set at 37°C (Coy Laboratory Products, Ann Arbor, Michigan). This incubator uses an oxygen analyzer to monitor and maintain the selected chamber oxygen concentration. This oxygen concentration was maintained with a calibrated gas mixture of 95% nitrogen and 5% carbon dioxide.

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**Antibodies and matrices.** Native rat type I collagen was purchased from Calbiochem Corp. (La Jolla, CA) and human type IV collagen from Sigma Chemical Co. (St. Louis, MO). Human plasma fibronectin was obtained from GIBCO BRL. Laminin-1 was prepared from the Engelbreth-Holm-Swarm tumor as described previously (21). Laminin-5 was purified according to Marinkovich et al., as described previously (22). Mouse mAbs against  $\alpha 2$  (P1E6),  $\alpha 3$  (P1B5),  $\alpha 5$  (P1D6),  $\beta 1$  (P4C10), and  $\beta 4$  (3E1) integrin subunits were obtained from GIBCO BRL (23–26). A mouse mAb against the EGF receptor (clone 74) was obtained from GIBCO BRL. GB3 antibody, recognizing the  $\gamma 2$  chain of laminin-5, was purchased from Accurate Chemical and Science Corp. (Westbury, NY) (27).

**Keratinocyte migration assays.** Keratinocyte migration was assessed by the method of Albrecht-Buehler (28), as modified by Woodley et al. (17). Briefly, colloidal gold salts were immobilized on coverslips and covered with extracellular matrix components such as type I collagen, type IV collagen, laminin, or fibronectin. Second to fourth passage keratinocyte cultures were suspended and plated on the coverslips and allowed to migrate for 16 h. The cells were fixed in 0.1% formaldehyde in PBS and examined under dark field optics with a video camera attached to a computer equipped with a frame grabber. The computer analyzes 15 nonoverlapping fields in each experimental condition with NIH Image 1.6 and determines the percentage area of each field consumed by cell migration tracks, a so-called Migration Index. The lengths of individual tracks were also measured. Confirmation of a difference in migration as statistically significant requires rejection of the null hypothesis of no difference between mean migration indices obtained from replicate sets at the  $P = 0.05$  level with a Student's  $t$  test (29). All migration assays were performed at 0.2, 2, 9 (tissue normoxia), and 20% oxygen (incubator normoxia), respectively.

A second migration assay, the so-called scratch assay was performed according to Cha et al., as described previously (30). Keratinocytes ( $40,000/\text{cm}^2$ ) were plated in tissue culture dishes, and after 24 h of incubation, the confluent monolayer of cells was scratched in a standardized manner with a plastic apparatus to create a cell-free zone in each well, 2 mm in width. The medium was aspirated and replaced with keratinocyte medium with or without type I collagen (15  $\mu\text{g}/\text{ml}$ ) diluted in keratinocyte medium. The cells were then incubated under normoxic and hypoxic conditions for an additional 16 h. In vitro wound closure was monitored by the repopulation of the cleared area with cells over time. The amount of migration was quantitated by computer-assisted image analysis using NIH Image 1.6. The residual gap between the migrating keratinocytes was measured and expressed as a percentage of the initial total scratch area. Each experiment was repeated three times.

Under hypoxic conditions, the medium pH drops to 7.1 over an 18 h period (personal observation). In sterile wounds the pH is 7.0–7.2 (15). The pH of SFM under normoxic conditions is 7.5. To assess the effect of lowering the pH of keratinocyte medium on keratinocyte motility, neonatal human keratinocytes in low calcium serum-free KGM were plated at a density of 1,000 cells/ $\text{cm}^2$  onto a matrix of type I collagen at a concentration of 15  $\mu\text{g}/\text{ml}$ . The cells were allowed to attach for 2 h at 37°C, the medium aspirated and replaced with medium that had been pH adjusted from 7.5 by the step-wise addition of either HCl or lactic acid while the oxygen tension was held at 9% (tissue normoxia). The cells were allowed to migrate overnight, fixed with 0.1% formaldehyde and the migration index was then calculated. To circumvent the minor change in pH caused by hypoxia, keratinocytes were incubated in the colloidal gold migration assay under hypoxic conditions in keratinocyte medium buffered with 10 mM Hepes to equilibrate the pH at a constant level of 7.5, and the migration assay was allowed to continue, as described above.

**Protein synthesis.** To compare total protein synthesis by keratinocytes under normoxic and hypoxic conditions, subconfluent numbers of cells were plated onto type I collagen (15  $\mu\text{g}/\text{ml}$ ) or no matrix and incubated under normoxic (20%) or hypoxic (2%) conditions for 16 h, in methionine-free KGM (Clonetics) in the presence of [ $^3\text{S}$ ]me-

thionine (ICN Biomedicals, Inc., Irvine, CA)—10  $\mu\text{Ci}/1.5$  ml. Two different labeling schedules were used. The label was added immediately after keratinocyte attachment and incubated for 16 h, or the cells were incubated in normoxic or hypoxic conditions for 12 h and the label added 4 h before collection. In both methods, the conditioned medium was collected after a 16 h total incubation period. The cell layers and associated matrix were extracted. Aliquots (50  $\mu\text{l}$ ) of conditioned medium were precipitated in 10% TCA at 4°C for 10 minutes. The pellets were washed with 5% TCA ( $\times 3$ ) at 4°C to remove any nonprotein associated counts and dissolved in 1 N sodium hydroxide (100  $\mu\text{l}$ ) before addition of scintillation fluid (Sigma Chemical Co.). After sonication and centrifugation, the supernatant was collected. Aliquots were added to the scintillation cocktail and counted on a Beckman Scintillation Counter. Keratinocytes were also plated at a density of 20,000 cells per  $\text{cm}^2$  under normoxic and hypoxic conditions for 16 h. The cells were extracted in 0.8 ml of 0.125 M Tris HCl, pH 6.8, 2% SDS (sample buffer without bromophenol blue dye) and the protein concentration determined by the method of Lowry et al. (31).

**Western immunoblots for ezrin, radixin, and moesin.** Keratinocytes driven to migrate by type I collagen were subjected to normoxic and hypoxic conditions and evaluated for the relative expression of the lamellipodia associated proteins, ezrin, radixin, and moesin (32). Briefly, keratinocytes were plated sparsely (12,500/ $\text{cm}^2$ ) on type I collagen, and cultured for 18 h under normoxic or hypoxic conditions. The cell layers were washed in cold PBS three times and then 0.8 ml of 0.125 M Tris HCl, pH 6.8, 2% SDS (sample buffer) or 0.8 ml 10 mM Tris, pH 7.5, 140 mM NaCl, 1% Triton X-100 was added to the cell layers. The cell layer was scraped with a plastic policeman and the buffer collected. The extracts were vortexed for 1 min, sonicated for 30 s, and centrifuged at 100,000 g for 15 min. The soluble supernatant was collected and the protein concentration determined according to Lowry et al. (31). After the protein determinations, bromophenol blue dye, 5 mM DTT, 1 mM EDTA, and 1 mM PMSF were added to the sample buffer extracts. The Triton X-100 extracts were mixed with equal amounts of 2 $\times$  sample buffer containing bromophenol blue dye, 10 mM DTT, 2 mM EDTA, and 2 mM PMSF. The samples were boiled and equal amounts of protein were subjected to SDS-PAGE and Western immunoblotting according to Towbin et al. (33). Identical nitrocellulose strips were incubated with polyclonal antibodies to ezrin (pAs 90.3, dilution 1:1,000), radixin (pAs 92.4, dilution 1:1,000), and moesin (pAs 90.7, dilution 1:300) and a mAb to actin (1:10,000) for 2 h (34). The nitrocellulose strips were then incubated with secondary peroxidase conjugated goat anti-rabbit IgG at a dilution of 1:2,000 or peroxidase conjugated goat anti-mouse IgG at a dilution of 1:1,000 (Organon Teknika-Cappel, Durham, NC) for 1 h and the membrane was developed using ECL (Amersham Corp., Arlington Heights, IL) or a four CN Membrane Peroxidase Substrate System (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). This experiment was repeated four times with four different keratinocyte donors (two adult, two neonatal).

**Immunofluorescence.** Keratinocytes were plated in TissueTek chamber slides (Nunc, Inc., Naperville, IL), on type I collagen at a density of 12,500 cells/ $\text{cm}^2$  and cultured under normoxic and hypoxic conditions for 18 h. The keratinocytes were immersed in periodate-lysine-paraformaldehyde fixative for 10 min at room temperature (35). The cells were washed several times with PBS to remove fixative, and then permeabilized and blocked by incubating in PBS with 3% BSA, 1% saponin, and 0.05% Na Azide for 10 min at room temperature. The cells were incubated with ezrin antibody, pAs 90.3 at a dilution of 1:200 in a humidified chamber for 2 h, then washed three times with PBS, 1% saponin, counterstained with a FITC-conjugated goat antibody to rabbit IgG (1:100 dilution) for 1 h (Organon Teknika-Cappel) and washed. The cells were then examined and photographed with a Zeiss epifluorescing immunofluorescence microscope at a magnification of 330.

**Western immunoblot for laminin-5 ( $\beta 3$  and  $\gamma 2$  chains) and fibronectin.** Normal human keratinocytes were plated on plastic cul-

ture dishes at a density of 17,500 cells/cm<sup>2</sup>. The cells were incubated under normoxic and hypoxic conditions for 18 h to allow adequate time for laminin-5 deposition. The matrix was extracted by serial extraction with Triton X-100 for 5 min, 2 M urea and 0.1 M NaCl for 10 min, and 8 M urea for 10 min, as described (36). The remaining substratum was extracted in sample buffer (0.125 M Tris HCl, pH 6.8, 2% SDS). All solutions contained 1 mM PMSF and 2 mM NEM (*N*-ethylmaleimide). The samples were separated by SDS-PAGE on a 5% acrylamide slab gel. The separated proteins were electrophoretically blotted to a nitrocellulose membrane, according to Towbin et al. (33) and labeled with polyclonal antibodies to the  $\beta 3$  chain and  $\gamma 2$  chain of laminin-5 at a dilution of 1:1,000 (generously supplied by Dr. Warren Hoeffler, Stanford University, Stanford, CA). Purified laminin-5 was used as a positive control. The nitrocellulose membranes were then incubated with secondary peroxidase conjugated goat anti-rabbit IgG at a dilution of 1:2,000 (as above) for 1 h and the membrane was developed using ECL. The experiment was repeated using three neonatal and two adult donors. A duplicate control blot with fibronectin as a positive control was labeled with a polyclonal IgG antibody to human fibronectin at a dilution of 1:1,000 (Organon Teknica-Cappel). The nitrocellulose membranes were then incubated with a secondary peroxidase conjugated rabbit anti-goat IgG at a dilution of 1:2,000 (Bio-Rad Laboratories, Richmond, CA) and developed using ECL (vide supra).

**Laminin-5 immunofluorescence.** Keratinocytes were cultured in TissueTek chamber slides (Nunc, Inc.) under normoxic and hypoxic conditions for 18 h. The keratinocyte monolayers were extracted by serial extraction as described above and the remaining deposited extracellular matrix was stained with GB3 antibody for laminin-5. The cells were fixed with ice-cold methanol, washed several times with PBS to remove fixative, and then permeabilized and blocked by incubating for 10 min at room temperature in PBS with 3% BSA, 1% saponin, and 0.05% Na Azide. The cells were incubated with GB3 antibody at a dilution of 1:10 in a humidified chamber for 1 h, then washed three times with PBS with 1% saponin, counterstained with FITC-conjugated goat antibody to mouse IgG (1:25 dilution) for 1 h (Kirkegaard & Perry Laboratories, Inc.), washed and the matrix deposited on the slides examined and photographed with a Zeiss immunofluorescence microscope at a magnification of 100.

**Fluorescent-activated cell sorting (FACS®).** To determine the profile of cell-surface integrins on keratinocytes migrating on matrix and subjected to normoxic and hypoxic conditions, FACS® analysis was performed according to Chen et al. (29) with antibodies to  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ ,  $\beta 1$ ,  $\beta 4$  integrin subunits or the EGF receptor. All primary antibodies were used at a dilution of 1:100. A FITC conjugated anti-mouse secondary antibody purchased from Jackson ImmunoResearch Labs (Bar Harbor, ME) was used as the secondary antibody at a dilution of 1:200.

**Zymography and immunoblot for 92 kD collagenase.** Normal human keratinocytes were plated on plastic and type I collagen at a density of 17,500 cells/cm<sup>2</sup>. The cells were incubated under normoxic and hypoxic conditions for 18 h. Gelatinolytic proteinases were assayed by gelatin-substrate enzymography (37). Type I collagen heated at 56°C for 20 min was incorporated into a 10% acrylamide gel. Aliquots of medium (15  $\mu$ l) were prepared for electrophoresis without heating or reduction, after concentration  $\times 5$  with Centricon-10. After SDS-PAGE, SDS was removed from the gels by 2.5% (vol/vol) Triton X-100 washes (2  $\times$  20 min), and the gels incubated in assay buffer (15 mM Tris-Cl, pH 7.4, 5 mM CaCl<sub>2</sub> with 1 mM PMSF and 2 mM NEM) at 37°C for 24 h. The reaction was stopped by staining the gels with Coomassie Brilliant Blue. Gelatinolytic activity was detected as clear bands against the aqua-blue stained gelatin background.

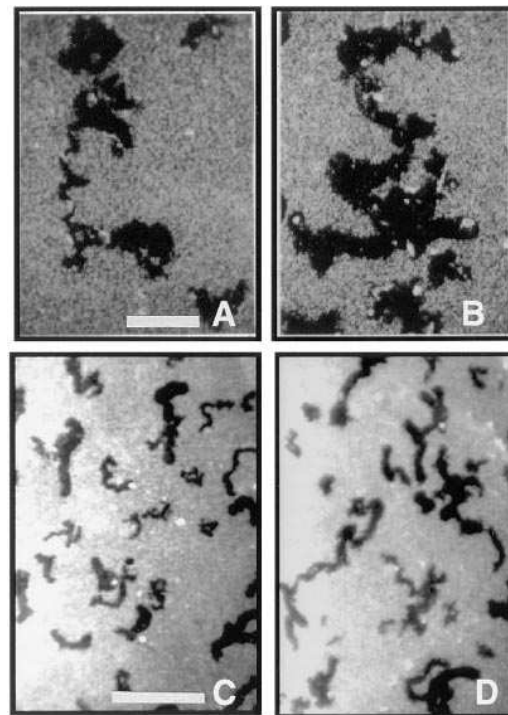
In order to determine the expression of the 92-kD type IV collagenase by another independent method, the samples were also subjected to SDS-PAGE on 10% acrylamide gels, the protein electrophoretically blotted to nitrocellulose membranes and labeled with a polyclonal peptide antibody to the 92-kD type IV collagenase (Triple Point Biologics, Forest Grove, OR) at a dilution of 1:1000 overnight

at 4°C. The nitrocellulose membranes were then incubated with secondary peroxidase-conjugated goat anti-rabbit IgG at a dilution of 1:2,000 for 1 h and developed using ECL.

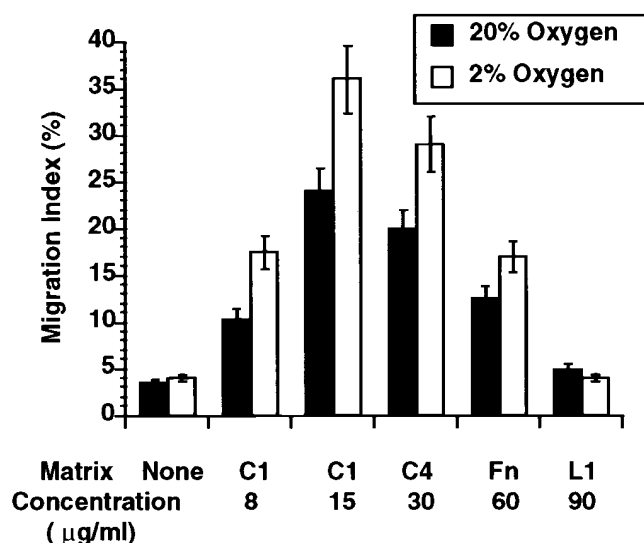
**Densitometry.** To quantitate the intensity of individual bands, the optical density and area were measured by scanning the film with a Bio-Rad BLS-670 densitometer (Bio-Rad Laboratories).

## Results

**Effect of hypoxia on keratinocyte migration.** In order to examine the effect of hypoxia on keratinocyte migration, we performed two distinct keratinocyte migration assays. As shown in Fig. 1, *A* and *B*, human keratinocytes juxtaposed to an extracellular matrix of type I collagen exhibit increased cellular migration under hypoxic conditions compared with normoxic conditions (compare 1 *A* [normoxic] with 1 *B* [hypoxic]). As shown in Fig. 2, the computer-generated Migration Indices increased 1.5–2.0-fold under hypoxic conditions versus normoxic conditions on type I collagen, type IV collagen and fibronectin using neonatal donor keratinocytes. A 1.5–2.0-fold increase in the Migration Indices under hypoxic conditions was also demonstrated using adult donor keratinocytes (data not shown). This hypoxia-driven enhanced motility was not observed when the cells were opposed to tissue culture plastic, albumin or laminin. Comparable Migration Indices were obtained under tissue normoxia (9% O<sub>2</sub>) and incubator normoxia (20% O<sub>2</sub>) and also under both hypoxic conditions (2% and 0.2% O<sub>2</sub>) (data not shown).



**Figure 1.** The effect of hypoxia on keratinocyte migration on extracellular matrix. (*A* and *B*) Migration of human keratinocytes on collagen I after 16 h of culture under normoxic conditions (*A*) and hypoxic conditions (*B*). Migration tracks appear black under dark field optics. Keratinocytes exhibit increased motility under hypoxic conditions. (*C* and *D*) Low power view of the migration tracks under normoxic (*C*) and hypoxic (*D*) conditions. (*A*) Bar, 100  $\mu$ m; (*C*) bar, 300  $\mu$ m.



**Figure 2.** Hypoxia increases keratinocyte motility. Hypoxic conditions increase keratinocyte motility 50–100% over normoxic conditions on optimal levels of collagen I (4 and 15  $\mu\text{g/ml}$ ), collagen IV (30  $\mu\text{g/ml}$ ) and fibronectin (60  $\mu\text{g/ml}$ ) using neonatal donors. These findings are representative of four different experiments using four donors. No statistically significant difference was noted on laminin or no matrix.

The motile keratinocytes also form longer tracks under hypoxic conditions as demonstrated in the lower power view of the dish surface in Fig. 1, *C* and *D*, normoxic versus hypoxic, respectively. As shown in Table I, the mean track length increased by  $\sim 40\%$  under hypoxic conditions in two neonatal donors and one adult donor when compared with identical cells migrating under normoxic conditions for exactly the same time.

In the *in vitro* wound assay, keratinocytes were plated densely in tissue culture wells and after 24 h a standardized scratch was made through the confluent monolayer as described previously (30). The keratinocytes from the cut edge of the scratch were then allowed to migrate for 12 h under normoxic or hypoxic conditions on type I collagen (15  $\mu\text{g/ml}$ ) or

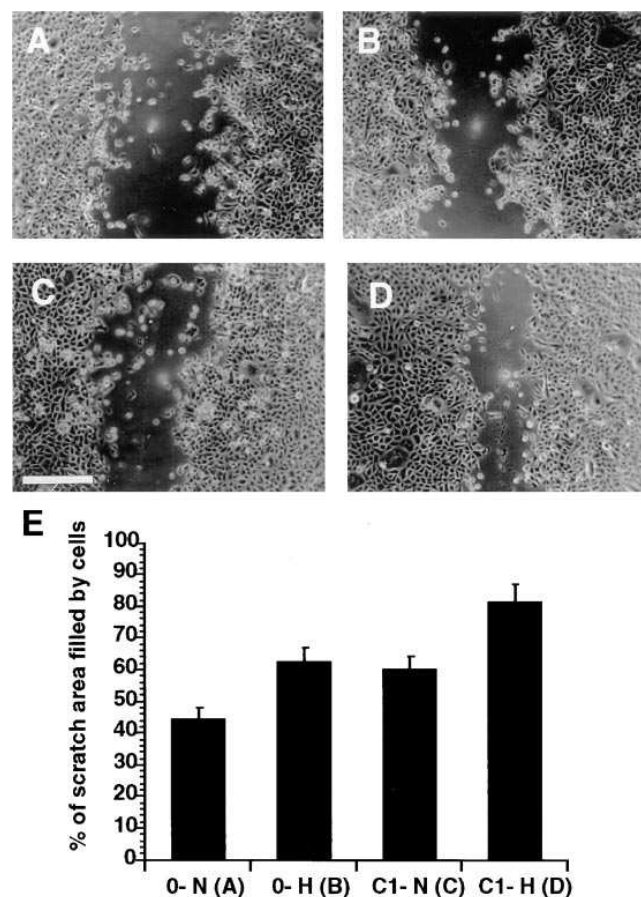
**Table I.** The Effect of Hypoxia on Migrating Human Keratinocyte Track Length

	Normoxia (20% O <sub>2</sub> )	Hypoxia (2% O <sub>2</sub> )
Donor 1	163.8 $\pm$ 21.2	221.1 $\pm$ 19.3
Donor 2	158.8 $\pm$ 19.1	234.2 $\pm$ 24.5
Donor 3	149.9 $\pm$ 16.4	220.3 $\pm$ 20.9

Neonatal human keratinocytes in low calcium serum-free SFM were plated at a density of 1,000 cells/cm<sup>2</sup> onto a matrix of collagen I at a concentration of 15  $\mu\text{g/ml}$ . The cells were allowed to attach for 2 h at 37°C and then incubated under normoxic and hypoxic conditions. The cells were allowed to migrate overnight, fixed with 0.1% formaldehyde and the length of individual keratinocyte tracks was calculated by computer-aided image analysis. As shown above, the length of the migration tracks is clearly increased under hypoxic conditions. Values are mean $\pm$ SEM of 10 tracks of three donors in micrometers.

no matrix, and photographed. The residual scratch area between the inwardly migrating keratinocytes from the edges of the scratch was measured as described in Methods. As shown in Fig. 3, the keratinocytes migrated inwardly and covered a greater area of the scratch under hypoxic conditions (Fig. 3, *B* and *D*) compared with normoxic conditions (Fig. 3, *A* and *C*). Further, the increased keratinocyte motility under hypoxic conditions relative to normoxic conditions was observed when the cells were apposed to both type I collagen and tissue culture plastic. As shown in Fig. 3 *E*, keratinocytes on plastic under normoxic conditions (*A*) migrated to fill 44 $\pm$ 2% of the scratch area. The filled area increased to 62 $\pm$ 4% under hypoxic conditions (*B*). Keratinocytes migrating on type I collagen under normoxic conditions filled 60 $\pm$ 3% of the scratch area (*C*), while the filled area increased to 82 $\pm$ 5% under hypoxic conditions (*D*).

**Effect of pH on keratinocyte motility.** Because hypoxia is associated with a lower pH of 7.1 in the culture medium (our observation), we sought to determine if a lower medium pH itself could influence keratinocyte motility as a variable indepen-



**Figure 3.** Effect of hypoxia on *in vitro* wound closure with and without collagen I. An *in vitro* wound was introduced in confluent cultures of normal human keratinocytes, as described in Methods. The cells were incubated without (*A* and *B*) or with collagen I 15  $\mu\text{g/ml}$  (*C* and *D*) and incubated under normoxic (*A* and *C*) or hypoxic conditions (*B* and *D*). After 12 h, the cells were photographed under phase-contrast microscopy. Bar, 200  $\mu\text{m}$ . The percent of the scratch area was calculated by image analysis and expressed as the mean $\pm$ SEM of three observations (*E*).

dent from hypoxia. To determine if the enhanced motility observed under hypoxic conditions could be explained by altered pH, keratinocyte migration assays under normoxic conditions were performed with and without the presence of exogenously added hydrochloric acid and/or lactic acid which induced corresponding decreases in pH. Human keratinocyte viability and proliferation are not affected by a medium pH as low as 5.4 (38). Progressively lower pH conditions are associated with decreased keratinocyte migration on type I collagen (Table II). A pH of 7.1 does not affect keratinocyte motility on extracellular matrix. However, a decrease in medium pH to 6.1 significantly inhibits keratinocyte motility (Table II). To circumvent the minor change in pH caused by hypoxia, keratinocytes were incubated under hypoxic conditions, but the medium was buffered with 10 mM Hepes to maintain the pH at 7.5. As shown in Table II, the increase in keratinocyte migration under hypoxic conditions was still apparent.

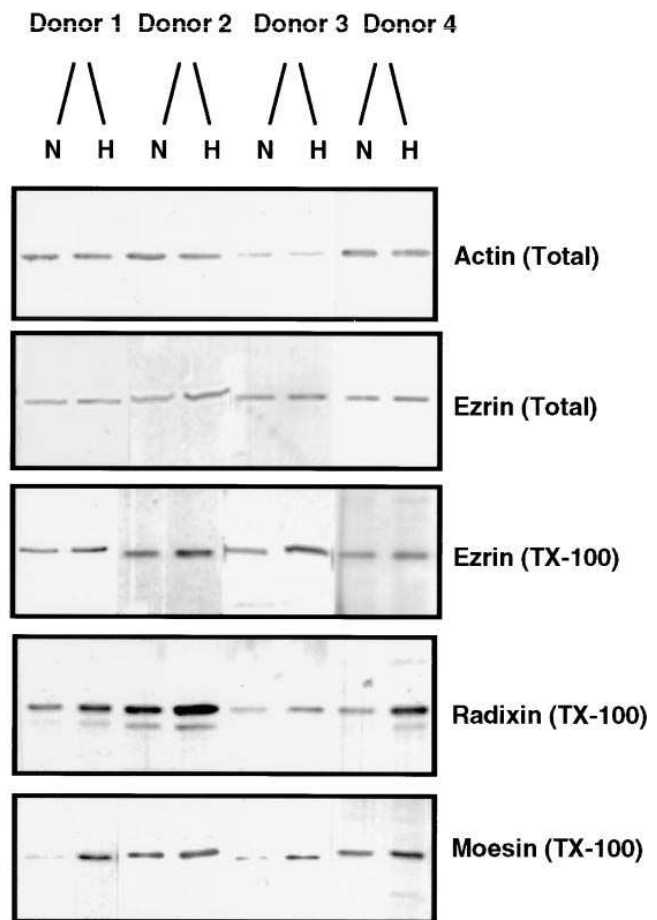
*Hypoxia does not affect protein synthesis.* Total protein synthesis and secretion by the keratinocyte cultures was not significantly altered under the conditions of the assay by hypoxia compared with normoxia. Regardless of the two schedules of adding [<sup>35</sup>S]methionine to the cultures, the TCA precipitable counts of incorporated methionine into newly synthesized proteins were essentially equal under both hypoxic and normoxic conditions in neonatal and adult donors (data not shown). The protein concentration of keratinocytes incubated under normoxic and hypoxic conditions was also assayed by the method of Lowry et al. (31), and no change in total cellular protein concentration was found (data not shown). These data are consistent with those of Herrick et al. who demonstrated recently that dermal fibroblast total protein synthesis is similar under normoxic and hypoxic conditions (39).

*Hypoxia-induced redistribution of ezrin, radixin, and moesin.* Ezrin, radixin, and moesin (ERM proteins) are constitutive proteins of epithelial cells that play a role in the formation of lamellipodia, plasma membrane extrusions present in motile

**Table II. The Effect of Altering Environment pH on Keratinocyte Migration**

pO <sub>2</sub> Additive pH	9% O <sub>2</sub>		9% O <sub>2</sub> Hepes 10 mM MI	2% O <sub>2</sub> Hepes 10 mM MI
	HCl MI	Lactic acid MI		
7.5	28±4%	28±5%	29±4.5%	37±4%
7.1	28±4%	28±3%		
6.1	18±3%	17±4%		

Neonatal human keratinocytes in low calcium serum-free SFM were plated at a density of 1,000 cells/cm<sup>2</sup> onto a matrix of collagen I at a concentration of 15 µg/ml. The cells were allowed to attach for 2 h at 37°C, the medium aspirated and replaced with medium that had been pH adjusted from 7.1 by the step-wise addition of HCl and lactic acid while the oxygen tension was held at 9% (tissue normoxia). The cells were allowed to migrate overnight, fixed with 0.1% formaldehyde and the percentage of the total field area consumed by migration tracks was calculated (the Migration Index). As shown above, as the pH decreases, the migration index decreases on collagen I. To circumvent the minor change in pH caused by hypoxia, keratinocytes were incubated under hypoxic conditions, but the medium was buffered with 10 mM Hepes. The increase in migration under hypoxic conditions was still apparent. Values are mean±SEM of 15 different field calculations in three donors.



**Figure 4.** Western blot demonstrating expression of ezrin and actin (total protein), ezrin, radixin, and moesin (plasma-membrane associated or Triton X-100 soluble) under normoxic and hypoxic conditions. Ezrin ( $M_r = 85,000$ ), radixin ( $M_r = 81,000$ ), and moesin ( $M_r = 78,000$ ) are lamellipodia-associated proteins. Keratinocytes plated on collagen I were incubated under normoxic and hypoxic conditions for 18 h. Donor keratinocytes (1 and 3, neonatal; 2 and 4, adult) were extracted in sample buffer and equal amounts of protein were subjected to SDS-PAGE. A representative experiment using each donor is shown.

cells (32). Because we observed that hypoxia induced enhanced keratinocyte motility, we postulated that the hypoxia-driven increased motility might be associated with a redistribution or increased expression of ERM proteins and increased lamellipodia formation. Total protein and Triton X-100 soluble protein extracts of keratinocyte cultures under normoxic or hypoxic conditions for 24 h were prepared and the expression of the lamellipodia-associated proteins ezrin, radixin, and moesin compared by Western immunoblot analysis. Enhanced expression of the plasma membrane associated ezrin, radixin, and moesin (Triton X-100 soluble) under hypoxic conditions compared with normoxic cultures was reproducibly demonstrated in four different experiments using different donor keratinocytes (Fig. 4). Representative experiments are shown in Fig. 4 (*Ezrin TX-100*, *Radixin TX-100*, and *Moesin TX-100*). By relative densitometry quantitation, the upward modulation by hypoxia was at least 100% for radixin and moesin and ~75% for ezrin (Fig. 5). Total ezrin, radixin, and moesin expression

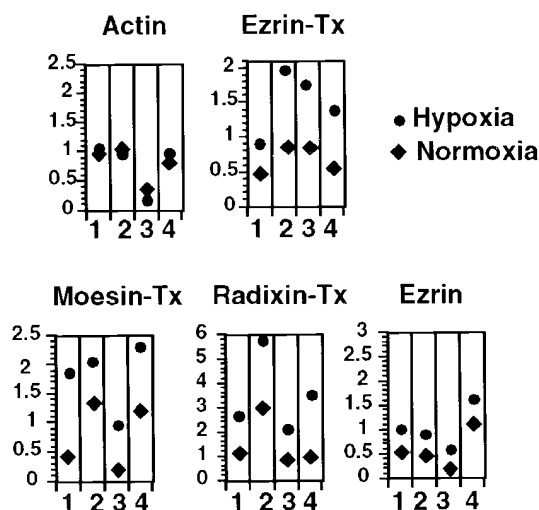


Figure 5. Densitometry values of bands shown in Fig. 4. To quantitate the intensity of individual bands, the optical density and area of each band were measured by scanning the film with a Bio-Rad BLS-670 densitometer. Each value represents the  $OD \times \text{area (mm}^2\text{)}$  of an individual band.

were also upregulated under hypoxic conditions in two neonatal and two adult donors, however, this upregulation was not as evident as the upregulation in the plasma membrane-associated fraction (data not shown for moesin and radixin, Fig. 4; ezrin labeled *Ezrin Total*). By relative densitometry quantitation, the upward modulation by hypoxia was 50% or greater of the normoxic condition in four identical experiments (see Fig. 5, *Ezrin*). We observed negligible change in the total actin expression under normoxic or hypoxic conditions (Figs. 4 and 5, *Actin Total* and *Actin*, respectively).

To examine more directly the morphology of keratinocyte lamellipodia under normoxic and hypoxic conditions, we immunolabeled keratinocytes migrating on type I collagen with a monospecific antibody to ezrin under varying oxygen tensions and then examined and photographed the cells. Representative microscopic fields are shown in Fig. 6 of keratinocytes migrating on type I collagen under normoxic and hypoxic conditions stained with ezrin antibody. As noted in the figure, the apron and the protruding lamellipodia become more elongated and more prominent under hypoxic conditions when compared with normoxic conditions (compare Fig. 6, A and B). This direct observation of enhanced lamellipodia formation being induced by hypoxia is concordant with the increased expression of the ERM proteins and redistribution of these proteins to the plasma membrane under hypoxic conditions.

**Effect of hypoxia on laminin-5 secretion by keratinocytes.** Laminin-5 is an important matrix attachment ligand for cultured keratinocytes and has been shown to inhibit keratinocyte motility (40, 41). We hypothesized that hypoxia might decrease keratinocyte secretion of laminin-5 and make the cell less firmly attached to the substratum. This process of less substratum anchorage would allow the cells to exhibit enhanced motility. To examine this hypothesis, we assessed the level of secreted laminin-5 in keratinocyte cultures under normoxic and hypoxic conditions. Equal amounts of protein, extracted from cells under normoxic and hypoxic conditions and sub-

jected to Western analysis, were probed with polyclonal antibodies to the  $\beta 3$  and  $\gamma 2$  chains of laminin-5, a major adhesion molecule for human keratinocytes. As demonstrated in Fig. 7, A and B (lanes 4 and 5), keratinocyte secretion of laminin-5 was decreased under hypoxic conditions compared with normoxic conditions. The intracellular laminin-5 (lanes 2 and 3) was not affected by hypoxia. Shown in Fig. 7 C is a duplicate blot probed with a polyclonal antibody to fibronectin. One can see that there is no change in the intracellular (lanes 2 and 3) or secreted (lanes 4 and 5) fibronectin expression under hypoxic (lanes 3 and 5) conditions compared with normoxic conditions (lanes 2 and 4). Shown in Fig. 7 D are representative Western blots and densitometry values of the expression of the  $\gamma 2$  chain of laminin-5 (155 kD) under normoxic and hypoxic conditions of three donors (two adult and one neonatal), confirming the selective decrease in the secretion of laminin-5 under hypoxic conditions.

In addition to Western blot analyses, we wished to directly assess the expression of secreted laminin-5 by migrating human keratinocytes under normoxic and hypoxic conditions by immunolabeling the cells with a monospecific antibody to laminin-5. As shown in Fig. 8 A, keratinocytes secrete copious

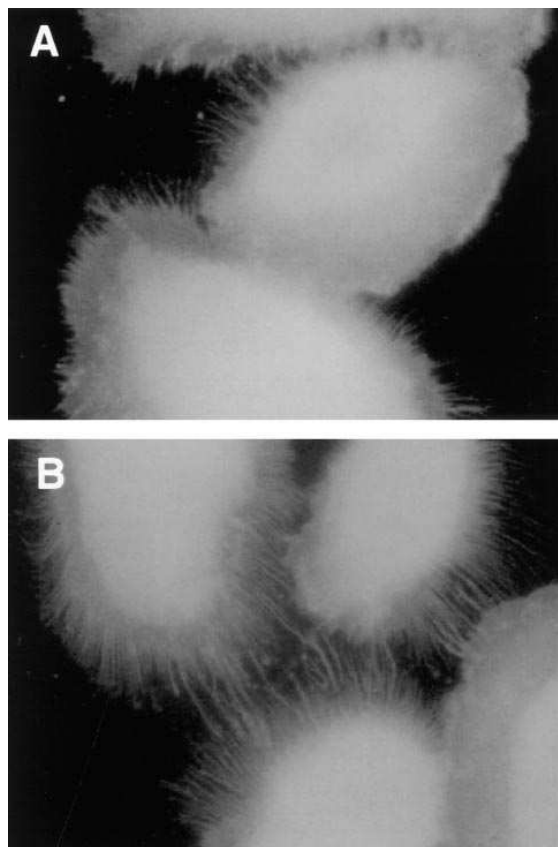
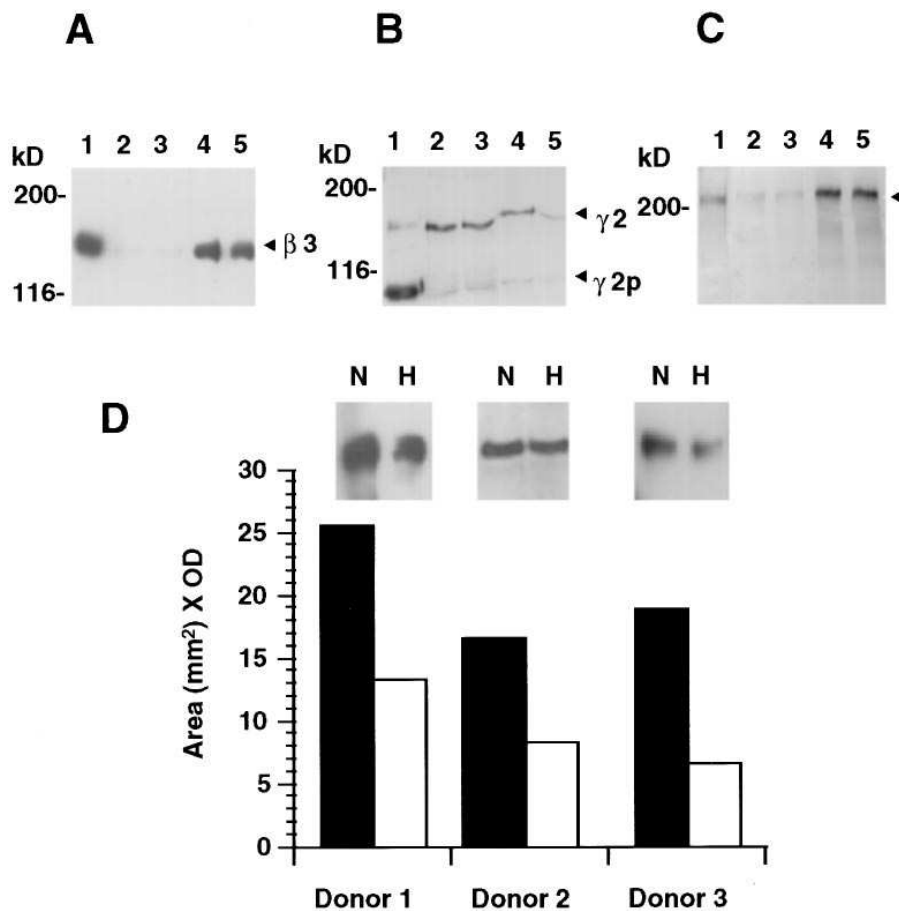


Figure 6. Immunofluorescence staining of ezrin under normoxic and hypoxic conditions. Human neonatal keratinocytes were plated on type I collagen, 15  $\mu\text{g/ml}$ , and incubated under normoxic (A) and hypoxic (B) conditions. The keratinocytes were washed with PBS, fixed and stained with a polyclonal antibody to ezrin and a FITC-conjugated secondary antibody. The cells were photographed with a Zeiss immunofluorescence microscope.  $\times 320$ .



**Figure 7.** Western blot demonstrating laminin-5 and fibronectin synthesis under normoxic and hypoxic conditions. Neonatal keratinocytes were plated on plastic tissue culture dishes at a concentration of 17,500 cells/cm<sup>2</sup>. The cells were incubated under normoxic and hypoxic conditions for 18 h. The cells were extracted as described in the text. Equal amounts of protein were subjected to SDS-PAGE and Western blotting. (A) The blotted proteins were labeled with an antibody to the  $\beta 3$  chain of laminin-5 (140 kD). (B) The blotted proteins were labeled with an antibody to the  $\gamma 2$  chain of laminin-5 (155 kD processed to 105 kD [ $\gamma 2p$ ]). (C) The blotted proteins were labeled with an antibody to fibronectin (220 kD). Lane 1 in each panel is the positive control. In each panel, lanes 2 and 3 are 2 M urea extracts from normoxic and hypoxic conditions, respectively. In each panel, lanes 4 and 5 are the remaining extracellular matrix after serial extraction from normoxic and hypoxic conditions, respectively. Under hypoxic conditions secretion of the  $\beta 3$  and  $\gamma 2$  chains of laminin-5 is decreased, however fibronectin remains unchanged. Protein bands were quantitated by densitometry.  $n = OD \times \text{area}$ .  $\beta 3$  chain: normoxic,  $n = 10.6$ ; hypoxic,  $n = 6.5$ .  $\gamma 2$  chain (155 kD): normoxic,  $n = 3.44$ ; hypoxic,  $n = 0.94$ . Fibronectin: normoxic,  $n = 7.828$ ; hypoxic,  $n = 8.02$ . (D) Laminin-5  $\gamma 2$  chain protein bands were quantitated by densitometry in three different experiments. Donors 1 and 2 (adult), donor 3 (neonatal). Solid bars, normoxia; clear bars, hypoxia.

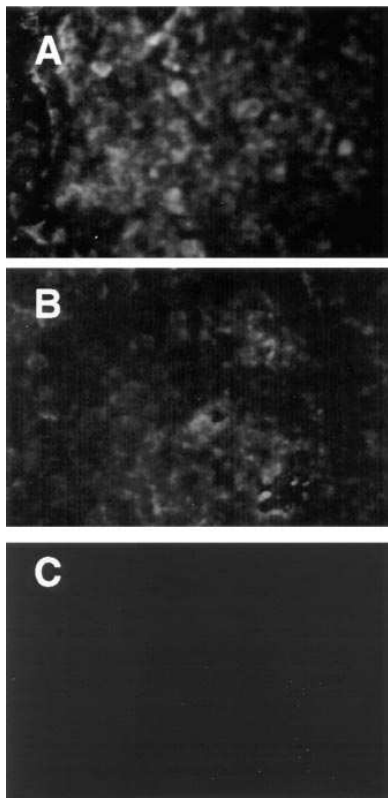
amounts of laminin-5 under normoxic conditions. This is visible as a sheet of irregular fluorescence (Fig. 8 A). Under hypoxic conditions, the amount of laminin-5 deposited by the keratinocyte is visibly diminished (Fig. 8 B). The negative control slide, stained with mouse IgG, (Fig. 8 C) demonstrated no fluorescent staining of the extracellular matrix.

**Effect of hypoxia on keratinocyte EGF and integrin receptors.** EGF and the EGF receptor play an important role in keratinocyte motility (29, 30, 42). Likewise, cell surface receptors called integrins that recognize extracellular matrices play a crucial role in keratinocyte migration (18, 29, 43, 44). Therefore, we sought to determine if hypoxic conditions altered the expression of keratinocyte integrins or the expression of the EGF receptor. We examined the cells by FACS<sup>®</sup> analysis (29) under both normoxic and hypoxic conditions. No quantitative differences in these receptors were associated with hypoxia versus normoxia when the cells were opposed to a matrix that supports motility, i.e., collagen I. Although the  $\alpha 3\beta 1$  integrin plays a unique role in integrin-mediated keratinocyte migration and the  $\alpha 6\beta 4$  integrin induces keratinocyte adhesion to laminin-5 via hemi-desmosomes (36, 43), the enhanced migration of keratinocytes under hypoxic conditions cannot be explained by an alteration in integrin receptors. An EGF/TGF- $\alpha$ /EGF-receptor-mediated pathway is activated during human

wound repair (30, 45), however this signaling pathway does not appear to be induced during hypoxia.

**Effect of hypoxia on gelatinase B expression.** Type I collagen (37), dibutyl cyclic AMP (46), and hepatocyte growth factor (47) increase keratinocyte migration in association with an increased expression of the 92-kD type IV collagenase by the cells. Therefore, we sought to determine if hypoxia-driven enhancement of keratinocyte motility was associated with an alteration in the expression of keratinocyte-derived collagenases. The 72- and the 92-kD type IV collagenases were readily detectable in conditioned medium of keratinocytes under normoxic and hypoxic conditions by zymography and Western blotting. By densitometry analysis of both zymograms and Western blots, we found that keratinocytes under hypoxic conditions demonstrated a 1.5–2-fold enhanced synthesis of the 92-kD collagenase, whether plated on type I collagen or tissue culture plastic when compared with normoxic conditions (Fig. 9). As demonstrated in Fig. 9 A, lanes 2 and 3 show the relative expressions of the 92- and the 72-kD gelatinases secreted by human keratinocytes migrating on a matrix of type I collagen under normoxic (lane 2) and hypoxic (lane 3) conditions. When one compares lane 2 with lane 3 in Fig. 9, one notes an enhancement of the 92-kD band corresponding to the 92-kD gelatinase, while the 72-kD bands, corresponding to the 72-kD





**Figure 8.** Immuno-staining of keratinocyte extracellular matrix under normoxic and hypoxic conditions. Keratinocytes were cultured in TissueTek chamber slides (Nunc, Inc.) under normoxic and hypoxic conditions for 18 h. The keratinocyte monolayers were extracted by serial extraction as described in the text and the remaining deposited extracellular matrix was stained with GB3 antibody for laminin-5, followed by a FITC-conjugated antibody. The cells were photographed with a Zeiss immunofluorescence microscope.  $\times 92$ .

gelatinase, are of similar density in both lanes. The enhanced expression of the 92-kD gelatinase by hypoxia compared with normoxia is more notable when the keratinocytes are apposed to tissue culture plastic (lanes 4 and 5) under the two different oxygen tensions than when apposed to a collagen substratum (lanes 2 and 3). Also, as noted in Fig. 9A, the increased expression of the 92-kD collagenase was selective because the 72-kD enzyme, readily visualized in the same zymogram (*lower arrow*), was not altered by hypoxia. These zymogram data were confirmed at the protein level by Western blotting (Fig. 9B). These experiments were repeated using two neonatal donors and one adult donor and the upregulation of the 92-kD type IV collagenase was a consistent finding.

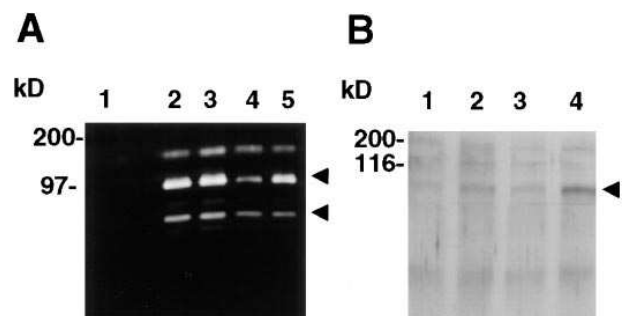
## Discussion

The human epidermis is an avascular tissue. Oxygen and other nutrients that feed the avascular epidermis are derived from a plasma filtrate from the dermal blood vessels. When a cutaneous wound occurs, the first event is coagulation of the blood within the dermal vessels. Therefore, the keratinocytes at the cut edge of the wound are likely to face acute hypoxia. Hypoxia may promote certain aspects of the wound healing process. For example, hypoxia has been shown to promote collagen synthesis in wounds (2). Likewise, hypoxia potentiates angiogenesis (48, 49) and promotes TGF- $\beta$ -mediated deposition of collagen (50–52).

In this study, we directly measured human keratinocyte motility on extracellular matrices under normoxic and hypoxic conditions in two independent migration assays. The colloidal gold assay measures the migration of single keratinocytes ap-

posed to a matrix. In contrast, the *in vitro* scratch assay measures the migration of a confluent sheet of keratinocytes over matrix. We found that hypoxia significantly enhanced keratinocyte motility on collagens and fibronectin compared with normoxic conditions. The pro-migratory effect of hypoxia is comparable in potency to soluble factors like EGF, TGF- $\alpha$ , and IL-1 $\alpha$  (30, 53). This result is counter to the notion that reduced oxygen tension has a negative effect on wound healing (3–5). While hypoxia may not be beneficial for overall wound healing, certain elements of the wound healing process such as re-epithelialization may be initiated and promoted by hypoxic conditions.

Occlusive dressings promote the early re-epithelialization of skin wounds (8–12, 13). Varghese et al. (15) demonstrated that the oxygen tension beneath occlusive dressings covering human skin wounds is very low, and yet these dressings promote re-epithelialization when used within the first 24 h after wounding (8–12, 13). Ambient oxygen tension at the surface of chronic wounds without occlusive dressings has been measured and found to be 18–21 mmHg ( $\sim 3\%$ ) (4, 54). In contrast, the oxygen tension of the wound surface beneath semioclusive wound dressings was found to be negligible (15). Therefore, semi-occlusive dressings promote hypoxic conditions at the wound surface where the advancing tongue of re-epithelializing keratinocytes is located. It is conceivable, then, that wound hypoxia induced by semioclusive wound dressings may ex-



**Figure 9.** (A) A representative zymogram demonstrating secretion of the 72-kD and the 92-kD type IV collagenases under normoxic and hypoxic conditions. Gelatin zymography was performed on proteins from conditioned medium from migrating keratinocytes incubated under normoxic and hypoxic conditions on either a collagen I matrix (lanes 2 and 3) or no matrix (lanes 4 and 5). Note that the large prominent white band running just below the 97-kD marker is increased in lanes 3 and 5 (hypoxic conditions) compared with lanes 2 and 4 (normoxic conditions). The 72-kD type IV collagenase is not altered by hypoxia. For the purpose of densitometry, a positive image of the zymogram in A was prepared and the relative expression of the bands quantitated by densitometry.  $n = OD \times \text{area}$ . The values for the 92-kD band were 19.41 (lane 2 = normoxic cells on collagen); 26.98 (lane 3 = hypoxic cells on collagen); 12.39 (lane 4 = normoxic cells on no matrix); and 20.67 (lane 5 = hypoxic cells on no matrix). (B) Western blot of 92 kD type IV collagenase of conditioned medium from keratinocyte cultures under normoxic and hypoxic conditions on no matrix (lanes 1 [normoxic] and 2 [hypoxic]) and collagen I (lanes 3 [normoxic] and 4 [hypoxic]). The relative expression of the bands was quantitated by densitometry. The values for the 92-kD band were 0.71 (lane 1 = normoxic cells on no matrix); 1.43 (lane 2 = hypoxic cells on no matrix); 0.73 (lane 3 = normoxic cells on collagen I); 1.88 (lane 4 = hypoxic cells on collagen I).



plain why re-epithelialization is enhanced beneath this type of dressing. Acute hypoxia may be a potent, natural signal for the marginal keratinocytes to migrate out over the wound bed and cover the provisional matrix of fibronectin, fibrin, and collagen. Our finding that hypoxia promotes keratinocyte motility provides experimental justification for the clinical use of semi-occlusive wound dressings.

The mechanisms of keratinocyte motility are not known, but Odland and Ross (55) demonstrated that lamellipodia formation occurs when human keratinocytes become migratory during reepithelialization. Ezrin, moesin, and radixin are constitutive proteins involved in the formation of lamellipodia (32, 34). Recently, ezrin and moesin have been shown to bind to actin (34, 56). Moesin and radixin are upregulated in experimental glomerulonephritis during mesangial cell repair after injury and ezrin plays a role in hepatocyte growth factor-mediated migration in epithelial cells (57, 58). These data are the only evidence to date linking these cytoskeletal proteins to motility. In this study, we show that human keratinocytes stimulated to migrate by hypoxia exhibit increased expression of lamellipodia-associated proteins and redistribute them to the plasma membrane. To determine whether the alterations in ERM expression and distribution were hypoxia or motility-induced, we examined these variables in confluent keratinocyte cultures held under normoxic and hypoxic conditions. Changes in the ERM proteins were not apparent (data not shown), suggesting that the changes seen in subconfluent cultures under hypoxic conditions are secondary to motility rather than hypoxia.

Laminin and laminin isoforms, such as laminin-5, inhibit human keratinocyte motility (17, 41). Laminin-5 is a powerful matrix attachment factor for human keratinocytes which mediates cell adhesion via integrins  $\alpha 3 \beta 1$  in focal adhesions and  $\alpha 6 \beta 4$  in hemidesmosomes (36, 40). In stable unwounded skin, the nonmigratory basal keratinocytes are resting on the lamina lucida, a 35-nm zone rich in laminin isoforms. In contrast to laminin isoforms, collagens and fibronectin in the wound bed provide keratinocytes with matrices that are known to strongly promote keratinocyte motility (59). In skin wounds, the re-expression of laminin within the neobasement membrane zone occurs at the time keratinocytes stop migrating (60). In this study, we found that hypoxic conditions induced a decreased expression of laminin-5 (Fig. 7). The inhibition of laminin-5 by hypoxia could conceivably perturb the keratinocytes' intrinsic motility brake, make the keratinocytes less anchored to their substratum, and allow the cells to exhibit enhanced motility.

Matrix receptors on cell surfaces, called integrins, have been shown to play central roles in human keratinocyte motility on connective tissue components (18, 29, 39, 44). Nevertheless, the enhanced keratinocyte migration induced by hypoxia cannot be explained by an altered expression of integrins or the EGF receptor since FACS<sup>®</sup> analysis demonstrated similar integrin profiles regardless of the oxygen tension. Changes in integrin receptor expression may not be required for the increase in migration induced by hypoxia. The increased motility may require redistribution or increased amounts of ERM proteins at the cortical membrane or changes in cell-substratum adhesion that could be achieved by differences in assembly/disassembly of adhesion sites. Although, protein synthesis is unaltered by hypoxia, selective changes in protein expression could be secondary to a direct effect of hypoxia on transcription or mRNA stability and translation (61).

Another mechanism involved in cellular motility is the expression of metalloproteinases (62–66). We have shown previously that, like dermal fibroblasts, human keratinocytes synthesize and secrete type I and type IV collagenases (37, 65, 66). Moreover, these collagenases exhibit increased expression when the keratinocytes are migrating (37, 65). A matrix of interstitial collagen induces keratinocyte motility and the expression of collagenases (37, 62–64). In this study, we show that keratinocytes induced to migrate by hypoxia exhibit an upregulation of the 92-kD type IV collagenase while the 72-kD type IV collagenase remains unaltered (Fig. 9). The increased activity of the 92-kD collagenase under hypoxic conditions was apparent in conditioned medium from confluent and subconfluent keratinocyte cultures (data not shown) suggesting that hypoxia directly influences collagenase secretion. AP-1, a transcription factor important in collagenase production, is induced by hypoxia in endothelial cells (67). It is likely that the increased 92-kD collagenase activity observed in our study occurs by a similar mechanism. The role of collagenases in cell motility may be to degrade the endogenously synthesized matrix used by the cells for transient attachment, allow for cell-matrix disadhesion, and permit continued cellular motility.

Intuitively, one might consider hypoxic conditions unfavorable for wound healing. Hyperbaric oxygen and oximeters have been used to optimize the oxygenation of skin wounds (7). Nevertheless, the efficacy of such maneuvers is controversial. For example, we could find only one controlled study supporting the use of hyperbaric oxygen for chronic leg ulcers (68), while other studies suggested hyperbaric oxygen has little or no effect on wound healing (69, 70). In this study, we have shown that human keratinocytes migrating on connective tissue matrices exhibit enhanced motility when subjected to hypoxia. This observation is in accordance with the fact that keratinocytes at the margins of skin wounds are in a hypoxic environment and the observation that clinically-enhanced re-epithelialization occurs in skin wounds treated with semiocclusive dressings. Hypoxia may initiate human keratinocyte migration by several cellular mechanisms including (a) the promotion of lamellipodia formation; (b) the enhancement of collagenase expression that serves to allow for matrix disadhesion and continued migration; and (c) a decrease in cell-substratum attachment mediated by decreased expression of laminin-5, a potent matrix attachment factor for keratinocytes.

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