Potential role for laminin 5 in hypoxia-mediated apoptosis of human corneal epithelial cells

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SUMMARY

Laminin 5 functions to promote cell-matrix adhesion and therefore is hypothesized to abrogate apoptosis initiated through the loss of epithelial cell contact with extracellular matrix. Laminin 5 levels are decreased in epithelial cells cultured in a hypoxic environment. Exposure of epithelial cells to hypoxia may induce apoptotic pathways transmitted through changes in mitochondrial membrane potential. Using an apoptosis assay based on mitochondrial membrane integrity, the effect of hypoxia (2% oxygen) on human corneal epithelial cell viability was determined. Both a virally transformed corneal epithelial cell line and third passage corneal epithelial cells were resistant to hypoxia-mediated apoptosis for up to 5 days in culture. However, at 7 days in culture, a statistically significant increase in apoptosis was noted in hypoxic corneal epithelial cells compared to normoxic (20% oxygen) controls. Increased apoptosis in hypoxic epithelium at 7 days in culture correlated with decreased deposition of laminin 5 into the extracellular matrix, as determined by western blot analysis and immunofluorescence microscopy. Additionally, the extracellular processing of the α3 and γ2 chains of laminin 5 was negatively impacted by corneal epithelial cell exposure to hypoxia for 7 days. Treatment of human corneal epithelial cells cultured in 20% oxygen with function-inhibiting antibodies to laminin 5 for 2 or 3 days resulted in a statistically significant decrease in proliferation, and concomitant increase in apoptosis, compared with untreated normoxic controls. Based on these results, it appears that mechanisms of hypoxia-mediated apoptosis in human corneal epithelial cells may be initiated by the loss of processed laminin 5 in the extracellular matrix or by the loss of laminin 5-epithelial cell communication and transmitted through mitochondria.

Key words: Apoptosis, Corneal epithelium, Hypoxia, Laminin 5

INTRODUCTION

Apoptosis is a defined form of controlled, or programmed, cell death (Kerr et al., 1972; Wylie et al., 1980). Apoptosis can be distinguished from necrosis or other forms of cell disruption by several morphological characteristics, including the presence of plasma membrane blebbing and nuclear condensation. Degradation and fragmentation of DNA occur in response to apoptotic signals and are an accepted hallmark of this form of cell death (Arends and Wylie, 1991; Earnshaw, 1995). Specific biochemical factors are unique to apoptosis, including the activation of caspases (Thornberry and Lazebnik, 1998), release of cytochrome c from mitochondria (Liu et al., 1996) and mitochondrial depolarization (Green and Reed, 1998; Wadia et al., 1998; Kohler et al., 1999). PARP (poly(ADP-ribose) polymerase) is a highly conserved nuclear enzyme in eukaryotes. PARP cleavage may occur in the apoptotic response as a result of the activity of caspase 3 (Thornberry and Lazebnik, 1998).

In epithelial cells a diversity of signals and events can induce the apoptotic form of cell death, including disruptions in normal cell-extracellular matrix (ECM) interactions. Apoptosis specifically induced by a breach in cell-matrix adhesion has been termed anoikis (Frisch and Francis, 1994). In stratified epithelia such as skin, tongue and cornea, an adhesion complex mediates cell-extracellular matrix attachment. The structure consists of a cytoplasmic hemidesmosome, extracellular anchoring filaments (composed of laminin 5) and anchoring fibrils (composed of collagen type VII), and the basement membrane (Borradori and Sonnenberg, 1999). α6β4 integrin is physically associated with the hemidesmosome and is unique in that its cytoplasmic domain interacts with intermediate filaments as opposed to actin (Stepp et al., 1990). Laminin 5 (α3β3γ2) is a major adhesive ligand resident in the basement membrane and can function as an extracellular attachment protein for epithelium. It binds with high affinity to α6β4 integrin (Champliaud et al., 1996).

Laminin 5/α6β4 interactions are crucial for the assembly and maintenance of adhesion complex components because the protein pair forms the core of the hemidesmosome (Green and Jones, 1996**)**. The loss of hemidesmosomes in basal epidermal cells of transgenic mice lacking functional β4 integrin correlated to weak adhesion and cell degeneration attributed to apoptosis (Dowling et al., 1996). The targeted disruption of the *Lama3* gene, which encodes the α3 subunit of laminin 5, resulted in survival defects in homozygous null animals (Ryan et al., 1999). These authors (Dowling et al., 1996; Ryan et al., 1999) have suggested that laminin 5 provides a survival advantage for keratinocytes and that α6β4 integrin interacts with laminin 5 to mediate an unidentified signal essential for cell survival.

Hypoxia-mediated cell death had been presumed to occur by necrosis (Jozsa et al., 1981). Recent studies however have suggested that hypoxia can also induce apoptosis in epithelial cells (Bossenmeyer-Pourie and Daval, 1997; Volm et al., 1999). Upregulation of collagen type IV and fibronectin observed as a consequence of chronic hypoxia (Vyas-Somani et al., 1996; Kim et al., 1996; Berg et al., 1998) implies that cell-matrix adhesion may not be affected by available oxygen. By contrast, acute hypoxia resulted in a significant downregulation of cell surface integrins, CD44 and NCAM, and an associated decrease in cell adhesion in two human melanoma cell lines and a human adenocarcinoma line (Hasan et al., 1998). In human keratinocytes subjected to acute hypoxia the number of cell surface integrin receptors did not decrease, but laminin 5 secretion was significantly inhibited (O'Toole et al., 1997).

We hypothesize that a disruption in functional laminin 5 protein in the extracellular matrix of hypoxic corneal epithelial cells promotes apoptosis. To test this hypothesis, we analyzed the effect of hypoxia on corneal epithelial cell proliferation, programmed cell death and laminin 5 production. Our work demonstrates that human corneal epithelial cells subjected to chronic hypoxia undergo apoptosis and deposit less functional laminin 5 protein into the extracellular matrix. We further examined the role of cell-matrix integrity in hypoxia-induced apoptosis by disturbing adhesion with function blocking antibodies to laminin 5. Our observations suggest that compromised cell-matrix communication, via altered homeostatis of laminin 5 function, is one mechanism of hypoxia-induced apoptosis.

MATERIALS AND METHODS

Antibodies

Monoclonal antibody (mAb) GB3, specific for the γ2 chain of laminin 5, was purchased from Harlan Sera-Lab (Loughborough, UK). mAb D4B5, also directed against the γ2 chain of laminin 5, was purchased from Chemicon International (Temecula, CA). This antibody recognizes both the unprocessed (155 kDa) and processed (105 kDa) forms of the γ2 chain. mAb Clone 17, which is specific for the 145 kDa laminin 5 β3 chain, was purchased from Transduction Laboratories (Lexington, KY). mAb 10B5 was a generous gift from J. Jones (Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL). mAb 10B5 recognizes both the unprocessed (190 kDa) and processed (160 kDa) forms of the α3 chain of laminin 5. mAb P3H9-2, a function blocking antibody to laminin 5, was purchased from Chemicon. mAb C-2-10 to PARP [poly (ADP-ribose) polymerase] was purchased from Oncogene Research Products (Cambridge, MA).

Culture of human corneal epithelial cells

The transfected human corneal epithelial cell line 10.014 pRSV-T, referred to as HCE-T (Kahn et al., 1993) was a generous gift from S. Ward (The Gillette Company, Gillette Medical Evaluation Laboratories, Gaithersburg, MD). HCE-T were maintained in serum-free keratinocyte growth medium containing 0.1% bovine insulin, 0.1% human epidermal growth factor, 0.4% bovine pituitary extract and 0.1% hydrocortisone (KGM; Clonetics , San Diego, CA). HCE-T are viable until passage 20, in this study the cells were used between passage 16 and 17. Human corneal epithelial cells (HCEC) were obtained as cryopreserved tertiary cultures from young donors (Cascade Biologics, Portland, OR). HCEC were maintained in serum-free EpiLife medium containing human corneal growth supplement as supplied by Cascade. These cells were not passaged further, but were used as tertiary cultures for all studies.

For studies analyzing the effect of oxygen on cell behavior, one set of cells was maintained at 37°C and 5% CO_2 in a conventional humidified tissue culture incubator. The oxygen level under these conditions (20%) was defined as normoxic. A second set of cells was maintained at 37°C and 5% CO_2 in a humidified environmental chamber (Coy Laboratory Products, Ann Arbor, MI). The oxygen level under these conditions (2%) was defined as hypoxic. An oxygen analyzer was used to maintain the oxygen level at 2% by regulating the flow of a calibrated mixture of 95% nitrogen and 5% CO_2 into the chamber. Cells were maintained for up to 14 days depending on the experiment, with medium changes every other day.

Cell proliferation assay

HCE-T were seeded in 96-well uncoated tissue culture plates at an initial plating density of 2000 cells/well and incubated in either 2% or 20% oxygen for a total of 1, 3, 7, 10, 12 or 14 days. In a second experiment cells were incubated in 20% oxygen in the presence of 10 μg/ml mAb P3H9-2 to inhibit laminin 5 function. Cells were cultured for a total of 3 days, including treatment with the antibody for 2 days. As a control, cells were treated with 10 μg/ml IgG. Four hours before analysis on each of the indicated days 5-bromo-2′-deoxyuridine (BrdU) was added to each of the wells to a final concentration of 10 μM. Cells were fixed and DNA was denatured using reagents supplied with a cell proliferation ELISA kit (Roche Diagnostics, Indianapolis, IN). Cells were labeled with anti-BrdU peroxidase conjugate, washed, and incubated with color development substrate containing tetramethylbenzidine. The absorbance of the samples was measured at 450 nm wavelength (A_{450}). The result of the ELISA is represented as a graph of the mean absorbance (A_{450})±s.e.m. (in arbitrary units) as a function of time in culture and oxygen level. Thirty replicate wells of each time point and oxygen level were assayed for the ELISA (n=30). Statistical analysis using Student's two-tailed t-test with significance level of $P<0.05$ was used to compare proliferation as a function of time in culture and oxygen level.

Detection of apoptosis in cultured human corneal epithelial cells

Apoptosis in HCEC adherent to glass coverslips was assayed by determining mitochondrial integrity using the MitoLight™ Apoptosis Detection Kit (Chemicon). Cells were cultured in 2% or 20% oxygen for 3, 5 or 7 days. In a second experiment, cells were cultured in 20% oxygen in the presence of 10 μg/ml mAb P3H9-2 to block laminin 5 function. Cells were cultured for a total of 3 days including treatment with the antibody for 2 days. As controls, cells were cultured in the presence of 10 μg/ml control IgG. At each culture time point unfixed cells were incubated with the MitoLight™ reagent for 30 minutes at 37°C, as suggested by the kit protocol. The cells were placed on a microscope slide and observed immediately using a Zeiss Axiophot fluorescence microscope.

In healthy cells, the lipophilic cationic dye employed in the assay partitions to the cytoplasm and also accumulates in mitochondria, owing to its uptake by biochemically intact organelles. In apoptotic cells with altered mitochondrial membrane potential the dye is evenly distributed throughout the cytoplasm. Using filters to detect fluorescein and rhodamine, healthy cells are identified as containing red mitochondria against a green background of cytoplasmic dye. We scored any cell with at least one labeled mitochondria as viable. By contrast, apoptotic cells are uniformly green with no detectable red-labeled mitochondria. Identical fluorescein and rhodamine fields at 20× magnification were digitized (SPOT Diagnostics, Sterling Heights, MI) and the digital images were overlaid using MetaMorph software (Universal Imaging, West Chester, PA). Five images were captured per coverslip. The total number of cells per field and the total number of cells lacking any red-labeled mitochondria (i.e. fluorescent green only) were counted to calculate the percentage of

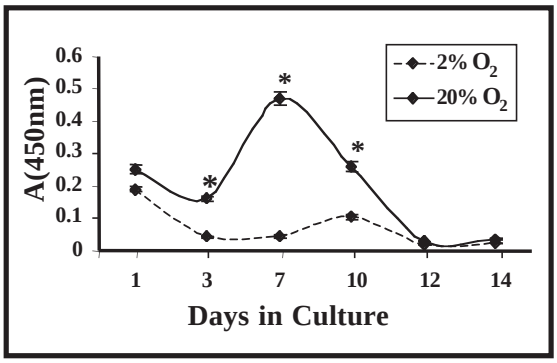

Fig. 1. Colorimetric BrdU cell proliferation ELISA of human corneal epithelial cells cultured under normoxic (20% O_2) or hypoxic (2% O_2) conditions. Proliferation is represented as the mean absorbance (A_{450}) in arbitrary units + s.e.m. (n=30 for each time point and oxygen level). For 3, 7 and 10 days in culture, proliferation is significantly higher (*, P<0.0001) in normoxic HCE-T compared with hypoxic HCE-T.

apoptotic cells as a function of culture conditions. Statistical analysis was performed using Student's two-tailed t-test and significance level P<0.05.

To confirm apoptosis, human corneal epithelial cells cultured for 7 days in 20% or 2% oxygen were lysed in gel sample buffer containing 60 mM Tris-HCl pH 6.8, 8 M urea, 1% SDS, 1% glycerol and 0.5% β-mercaptoethanol (Klatte et al., 1989). Protein concentrations were determined by the method of Henkel and Beiger (Henkel and Beiger, 1994) and 15 μg total cell protein were resolved using 7.5% SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose membrane and processed for western blot analysis using mAb to PARP at 1:1000 dilution. Immunoreactive proteins were detected and analyzed as described in the following section.

Western blot analysis of intracellular and extracellular laminin 5 content in cultured human corneal epithelial cells

HCE-T were cultured for 3 or 7 days in 2% oxygen or 20% oxygen. Cells and their deposited extracellular matrix were lysed in the urea/SDS gel sample buffer. 15 μg total cell protein was resolved using 7.5% SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose membrane and processed for Western blot analysis using mAb D4B5 to laminin γ2 chain (1:1000 dilution).

Immunoreactive proteins were detected using alkaline phosphatase-conjugated goat-anti mouse IgG (1:3000) as the secondary antibody coupled with the Immun-Star chemiluminescent protein detection system (BioRad Laboratories, Hercules, CA). Molecular weight markers obtained from BioRad were run with each gel and used to approximate the molecular weights of the immunoreactive proteins. The intensity of individual immunoreactive protein bands was determined by scanning the developed X-ray films and measuring the optical density and area of the bands using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Results of the densitometric analyses are represented in arbitrary units of the relative density of immunoreactive protein bands per μg total protein loaded on the gel. Western blot analysis was repeated five times with similar results.

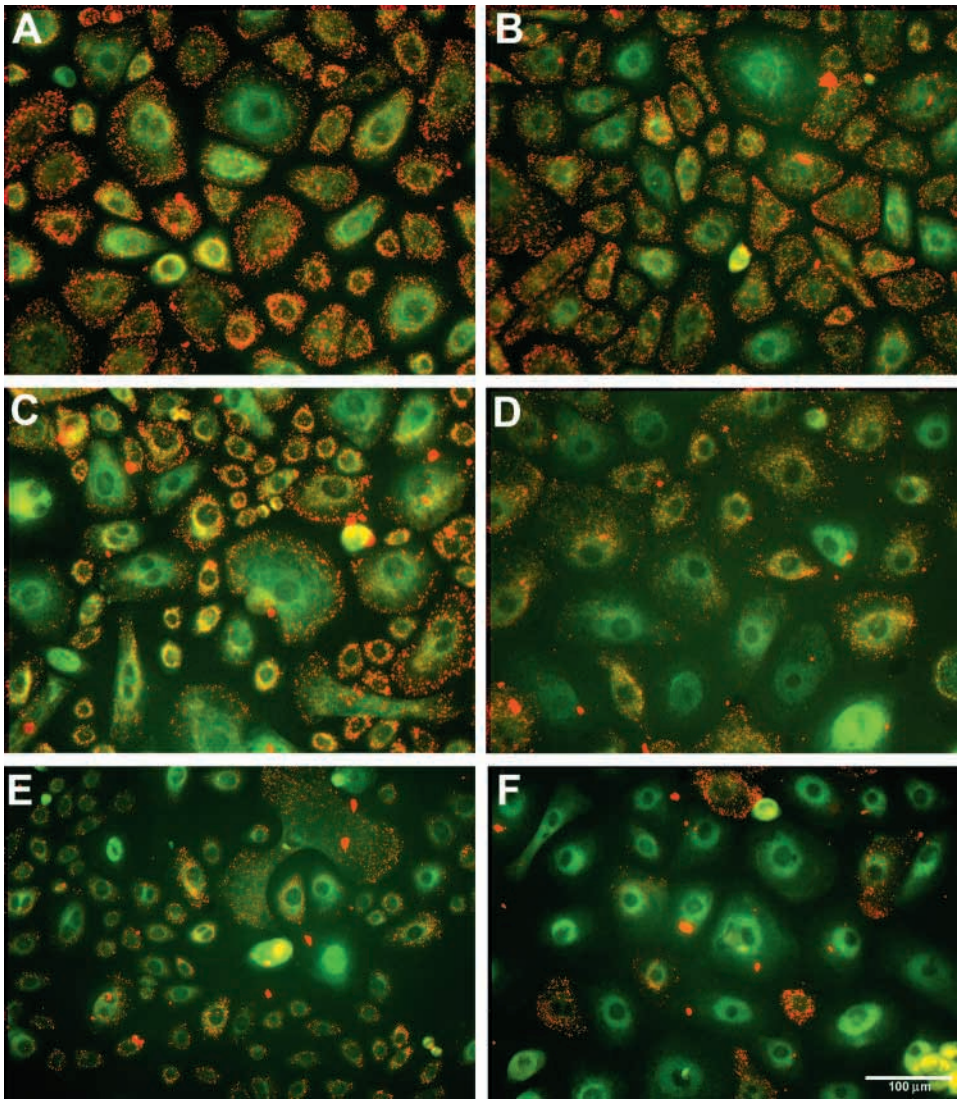

Fig. 2. Light microscopic analysis of mitochondrial membrane function in normoxic and hypoxic human corneal epithelial cells. HCEC were cultured for 3 days in 20% (A) or 2% (B) oxygen, 5 days in 20% (C) or 2% (D) oxygen, or 7 days in 20% (E) or 2% (F) oxygen before processing for apoptosis detection using the MitoLight™ technique. In each overlay in this Figure the green fluorescence represents cytoplasmic pools of a lipophilic cationic dye. The red fluorescence represents dye that has accumulated in mitochondria. Cells containing red-labeled mitochondria are scored as viable cells while those that lack labeled mitochondria and are uniformly green in color are scored as apoptotic. Note that at 5 and 7 days in culture, the normoxic cells appear to be smaller in size compared with the hypoxic cells.

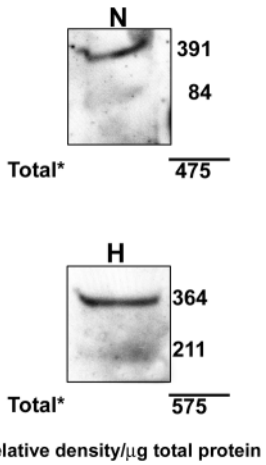

Fig. 3. Western blot and densitometric analysis of PARP content in normoxic and hypoxic human corneal epithelial cells. HCE-T were cultured for 7 days under normoxic (N) or hypoxic (H) conditions. 15 μg of cell lysates were resolved using 7.5% SDS-PAGE and processed for western blot analysis using mAb to PARP. Both the 115 kDa full-length PARP and the 89 kDa PARP cleavage product characteristic of apoptosis are detected by immunoblot. The relative density values of each PARP species determined by densitometry are indicated (in arbitrary units/μg total protein) to the right of each protein. The total of the densitometry readings is also indicated.

Western blot analysis of ECM deposited by human corneal epithelial cells

HCE-T, in wells of a 24-well tissue culture plate, were cultured at an initial plating density of 25,000 cells/well in 2% or 20% oxygen for 7 days. Cells were exposed to the different oxygen levels immediately following trypsinization and replating. The confluent epithelial cell layer was lysed in 0.1 N ammonium hydroxide (Langhofer et al., 1993). The ECM remaining on the culture substrate was washed extensively with sterile phosphate-buffered saline to remove all cell debris. 40 μl of gel sample buffer was used to solubilize the ECM in one well and the samples were loaded on to three different lanes of a 7.5% acrylamide gel. ECM from hypoxic and normoxic cell culture was loaded side-by-side to allow for a direct comparison of matrix composition. Western blot analysis using mAb 10B5 (1:3), Clone 17 (1:1000) or D4B5 (1:500), and densitometric analysis was completed as described. This western blot analysis was repeated five times.

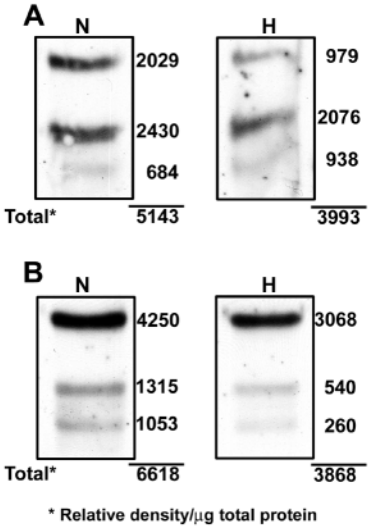

Fig. 4. Western blot and densitometric analysis of laminin 5 γ2 chain protein content in normoxic and hypoxic human corneal epithelial cells. HCE-T were cultured for 3 days (A) or 7 days (B) under normoxic (N) or hypoxic (H) conditions. 15 μg of cell lysates were resolved using 7.5% SDS-PAGE and processed for western blot analysis using mAb D4B5 to the γ2 chain. The unprocessed 155 kDa protein and processed 105 kDa and 80 kDa proteins are detected by immunoblot. The relative density values of each band are indicated (in arbitrary units/μg total protein) to the right of each immunoreactive protein. The total of the three densitometry readings is also indicated.

Immunofluorescence microscopy

To prepare HCE-T for immunofluorescence microscopic analysis, cells cultured on glass coverslips were fixed for 5 minutes in acetone at –20°C then washed in PBS for an additional 5 minutes. The fixed cells were probed with mAb GB3 following standard single-label direct immunofluorescence techniques (Klatte et al., 1989). The cells were examined using a Zeiss Axiophot fluorescence microscope. Fluorescein images at 20× magnification were captured and digitized

RESULTS

Effect of hypoxia on corneal epithelial cell proliferation and apoptosis

To assess the effects of oxygen on mitotic activity of human corneal epithelium a cell proliferation ELISA was performed. Human corneal epithelial cells (HCE-T) were cultured to confluence in 20% oxygen, trypsinized, replated and immediately placed into either a 2% or a 20% oxygen environment. Cells maintained in 20% oxygen (normoxic) demonstrated significantly higher (P<0.0001) levels of proliferation compared with those maintained in 2% oxygen (hypoxic) at 3, 7 and 10 days in culture (Fig. 1). Cells exposed to 20% oxygen exhibited a peak in proliferative capacity at 7 days in culture. By 12 and 14 days in culture however proliferation was equivalent regardless of the oxygen level. It is likely that the more prolonged culture periods resulted in stratified cell layers in the wells of the microtiter plate and thus inhibited proliferation even in normoxic cells.

To determine if decreased proliferation in hypoxic cultures was due to an imbalance of mitosis versus apoptosis, apoptosis was assessed using mitochondrial membrane function based on the uptake of a lipophilic cationic dye. Dye uptake by mitochondria, visualized as the accumulation of red fluorescence in the organelles against a green background of cytoplasmic dye, indicated a living cell. By contrast, mitochondria in apoptotic cells are incapable of accumulating the dye due to membrane depolarization. Red fluorescence cannot therefore be observed in apoptotic cells, and the cells appear uniformly green. No differences were observed in the apoptotic response to hypoxia between HCE-T and HCEC. Visual analysis of hypoxia-mediated apoptosis using HCEC are shown in Fig. 2.

After 3 days in culture, no major differences in mitochondrial dye uptake were noted between normoxic (Fig. 2A) and hypoxic (Fig. 2B) human corneal epithelial cells. Quantitative analysis of apoptosis was accomplished by scoring cells containing at least one red-labeled mitochondrion as viable. Cells lacking any red-labeled mitochondria, displaying a uniformly green appearance, were scored as apoptotic. This analysis demonstrated that after 3 days, 13% of the HCEC cultured in 20% oxygen and 10% of HCEC exposed to 2% oxygen were apoptotic (average of five fields counted).

After 5 days in culture, normoxic cells still contained numerous functional mitochondria (Fig. 2C). However, apoptotic cells were now evident in the hypoxic cultures (Fig.

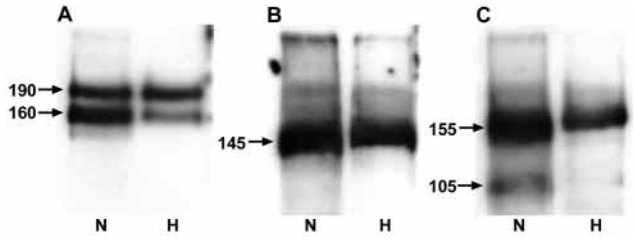

Fig. 5. Western blot analysis of laminin 5 protein levels in ECM produced and deposited by normoxic (N) or hypoxic (H) human corneal epithelial cells. The ECM was solubilized, separated by 7.5% SDS-PAGE and processed for Western blot analysis using mAb 10B5 to the laminin 5 α3 chain (A), mAb Clone 17 to the β3chain (B) or mAb D4B5 to the γ2 chain (C). The molecular weight of each of the immunoreactive proteins is indicated.

2D). Quantitative analysis of HCEC demonstrated a statistically significant difference in the number of apoptotic cells in normoxic versus hypoxic cultures after 5 days. In 20% oxygen 18% of HCEC were apoptotic. By contrast in 2% oxygen, 30% of the cells were apoptotic (average of five fields counted, $P<0.01$ compared with normoxic control).

After 7 days cells cultured under normoxic conditions still contained labeled mitochondria, with little evidence of extensive apoptosis (Fig. 2E). Indeed, the average percentage of apoptotic HCEC remained at 18%. However, extensive apoptosis was characteristic of hypoxic cells maintained in culture for 7 days (Fig. 2F). 70% of hypoxic HCEC were determined to be apoptotic at this time point in culture (average of five fields counted, $P<0.01$ compared with normoxic control).

To provide a quantitative analysis of apoptosis the degradation of PARP from the full-length 115 kDa to the 89 kDa fragment was assessed using western blot analysis. Although it was possible to visualize increased apoptosis using the mitochondrial assay in cultured corneal epithelial cells after 5 days, by densitometry increased PARP cleavage in hypoxic cells was not demonstrated until 7 days in culture (Fig. 3). At 7 days in culture, the 89 kDa degradation product accounted for 17% of the total PARP in normoxic cells, in contrast to 37% total PARP in hypoxic cells. An increase in the relative amount of the degraded PARP species in the hypoxic cell lysate suggests that a greater proportion of cells were apoptotic compared with normoxic controls.

Effect of hypoxia on laminin 5 content in corneal epithelial cells

In keratinocytes exposed to acute periods of hypoxia levels of secreted laminin 5 γ2 chain are reduced compared with control cells (O'Toole et al., 1997). To assess the effects of hypoxia on both the amount and the processing of laminin 5 γ2 chain in human corneal epithelial cells, western blot analysis was used. The biochemical analysis of laminin 5 content was performed using HCE-T because of the ease in obtaining large quantities of this cell type.

The γ2 chain of laminin 5 is extracellularly processed from 155 kDa to 105 kDa (Matsui et al., 1995; Goldfinger et al., 1998). Giannelli et al. (Giannelli et al., 1999) have also suggested that further processing to 80 kDa may occur. In HCE-T lysates containing both cellular and extracellular laminin 5 protein, all three molecular weight species of the γ2 chain were detected by western blot. Densitometric analysis showed that the total amount of all unprocessed and processed forms of γ2 chain was decreased in hypoxic HCE-T compared with normoxic HCE-T (Fig. 4). This held true for cells cultured for either 3 days (22% less total γ2 chain in hypoxic cells) or 7 days (42% less total γ2 chain in hypoxic cells). The ratio of unprocessed to processed forms of the chain also differed with respect to time in culture and oxygen level. After 3 days in culture, a greater proportion of γ2 chain was found to be in the processed forms in the hypoxic HCE-T (75% compared with 60% for normoxic HCE-T). By contrast, by 7 days in culture less of the processed forms of the chain were detected in hypoxic HCE-T (21% compared with 36% for normoxic HCE-T).

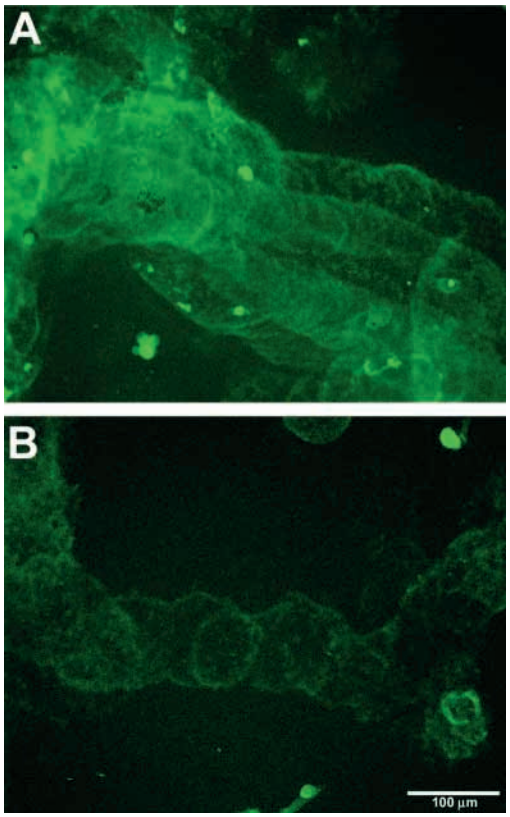

Fig. 6. Immunofluorescence microscopic analysis of laminin 5 localization in normoxic and hypoxic human corneal epithelial cells. HCE-T were cultured for 3 days in 20% oxygen (A) or 2% oxygen (B) before processing for immunofluorescence microscopy using mAb GB3 to the γ2 chain of laminin 5. Under normoxic conditions, the cells secrete and deposit a robust extracellular trail of laminin 5 protein that demarcates the path of movement of cells along the culture substrate (A). Note that the fluorescence intensity of the corresponding structures in the hypoxic cells is decreased (B), suggesting that less laminin 5 is deposited into the matrix.

The extracellular processed forms of the laminin 5 chains are vital to epithelial cell adhesion and survival. Therefore, in a second quantitative western blot analysis only the laminin 5 protein deposited into the ECM was examined (Fig. 5). Densitometric comparison of the relative levels of the three laminin chains reveals that after 7 days in culture 46% less total (unprocessed and processed forms) α3 chain, 22% less β3 chain and 57% less γ2 chain is deposited into the ECM by

hypoxic HCE-T compared with normoxic cells. Additionally, the relative percentages of the processed forms of the α3 and γ2 chains are decreased in hypoxic ECM compared with normoxic ECM. Specifically, in normoxic ECM, 59% of the α3 chain is in the processed form, but in hypoxic ECM 36% is in the processed form. In normoxic ECM, 29% of the γ2 chain is in the processed form but in hypoxic ECM 3% is in the processed form.

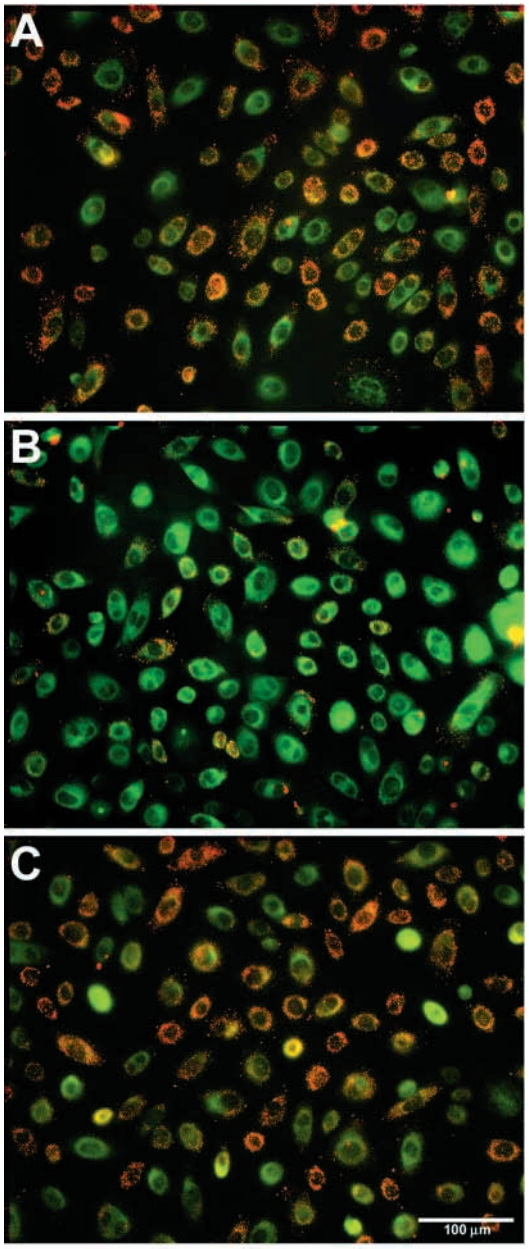

Fig. 7. Light microscopic analysis of apoptosis in normoxic human corneal epithelial cells. HCEC were cultured for 3 days in 20% oxygen. Cells in A represent the non-treated control. Cells in B were treated with function-blocking antibody to laminin 5 for 2 of those 3 days. Cells in C were treated with control IgG for 2 of those 3 days. All cells were processed for apoptosis detection using the MitoLight™ technique. Cells treated with function-blocking antibody to laminin 5 display a higher degree of apoptosis compared with untreated or IgG control cells.

Differences in the laminin 5 content of ECM deposited by hypoxic or normoxic HCE-T was also discernable by immunofluorescence microscopy using a mAb to the γ2 chain of laminin 5. This chain is unique to laminin 5; therefore. by using antibody probes to γ2 chain we are certain to be visualizing only the localization of the laminin 5 isoform. In normoxic cells robust extracellular trails of laminin 5 were observed (Fig. 6A). Laminin 5 trails have also been noted in other cell types (Zhang and Kramer, 1997). Hypoxic HCE-T were also capable of depositing an extracellular laminin 5 trail (Fig. 6B). However, the relative fluorescence intensity of the hypoxic trail structure was less than that of the corresponding normoxic trail, supporting the western blot analysis that suggests less laminin 5 protein is deposited into the ECM of hypoxic HCE-T. This observation agrees with that of O'Toole et al. (O'Toole et al., 1997), who reported diminished γ2 chain fluorescence in ECM deposited by cultured human keratinocytes.

Effect of perturbations of corneal epithelial cell-ECM interactions on apoptosis

To determine the effect of inhibiting laminin 5 function on apoptosis in otherwise normal cells, HCEC were cultured for 1 day to permit the cells to attach to the coverslip, then for an additional 2 days in the presence of a function-inhibiting antibody to laminin 5. Throughout this experiment the HCE-T were maintained in an atmosphere of 20% oxygen. Normoxic cultures of HCEC treated with antibody to laminin 5 (Fig. 7B) demonstrated more apoptotic cells compared with normoxic untreated (Fig. 7A) or IgG-treated controls (Fig. 7C). Quantitative analysis of HCEC confirmed a statistically significant increase in apoptotic cells in antibody-treated cultures. After 3 days in culture, 17% of the untreated cells were apoptotic compared with 40% apoptosis in the antibody-treated culture (average of five fields counted, $P<0.01$). Cells treated with function-inhibiting antibody to laminin 5 also demonstrated significantly less proliferative capability compared with IgG-treated controls (Fig. 8).

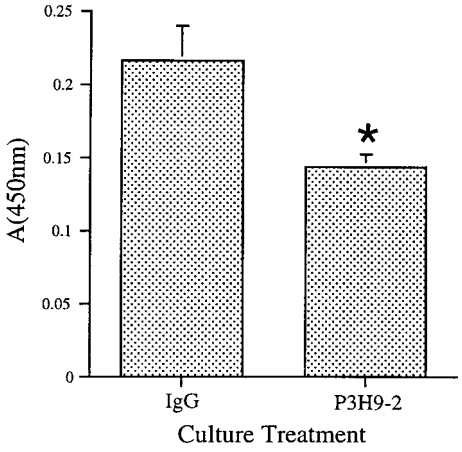

Fig. 8. Colorimetric BrdU cell proliferation ELISA of human corneal epithelial cells cultured under normoxic (20% O_2) conditions in the presence of function-blocking antibody to laminin 5 (P3H9-2) or control IgG. Proliferation is represented as the mean absorbance (A_{450}) in arbitrary units + s.e.m. (n=30 for each culture treatment). Proliferation is significantly lower (*, P=0.0004) in normoxic cells treated with laminin 5 antibody compared with control IgG.

DISCUSSION

Defects in mitochondrial function are thought to be involved in apoptotic mechanisms (Kroemer et al., 1997), and mitochondrial depolarization has been reported in several models of apoptosis (Lemasters et al., 1998; Wadia et al., 1998). Overexpression of the anti-apoptotic gene *Bcl2* in the rat pheochromocytoma cell line PC12 prevents hypoxia-mediated cell death, demonstrating that hypoxia can induce apoptosis (Shimizu et al., 1996). Levy et al. (Levy et al., 2000) have suggested that exogenous stimuli including hypoxia trigger an apoptosis pathway transmitted through mitochondria. O'Toole et al. (O'Toole et al., 1997) have shown that hypoxic human keratinocytes in culture secrete less laminin 5 γ2 chain. In cutaneous lesions produced by exposure to sulfur mustard, significant loss of laminin 5 from the basement membrane and apoptosis were observed concurrently (Smith et al., 1997). The studies in the present report were designed to bridge the gap between these independent investigations. We provide evidence that hypoxic human corneal epithelial cells in culture deposit less laminin 5 protein into the ECM substrate. The loss of homeostatic levels of laminin 5 in the ECM subsequently correlates to apoptosis in the hypoxic cells.

Human corneal epithelial cells appear to be resistant to deleterious effects of hypoxia for up to 5 days in culture, as we did not detect significant changes in mitochondrial function or relative percentages of apoptotic cells until day 5. PARP cleavage, characteristic of the initiation of the apoptotic pathway, was not evident until 7 days culture in the hypoxic environment. Hypoxia-mediated apoptosis occurred in human cultured trophoblasts after 24 hours in culture (Levy et al., 2000). Treatment with EGF to enhance trophoblast differentiation conferred a resistance to hypoxia-mediated apoptosis. These authors suggested that differentiated cells might be more resistant to hypoxia. However, while adult ventricular myocytes demonstrated evidence of apoptosis after 1 hour of hypoxia (de Moissac et al., 2000), neonatal ventricular myocytes became apoptotic only after 12-24 hours of hypoxia (Long et al., 1998; Tanaka et al., 1994). These results suggested that neonatal myocytes are more resistant to hypoxia. HCE-T are an SV-40 virally transformed cell line derived from adult human cornea, and HCEC are a tertiary culture derived from normal young human donors. The apoptotic response to chronic hypoxia appears to be similar in both cell types. We cannot conclude, based on the conflicting reports from other investigations, that the differentiation state of HCE-T or HCEC alone explains the ability of human corneal epithelial cells to resist the apoptotic effects of hypoxia in the short term. However, corneal epithelial cells are unique in that they typically exist and thrive in an avascular environment, deriving nutrients and oxygen from the tear film in lieu of an extensive vascular bed. Corneal cells may thus be predisposed to survival in unfavorable environments, explaining why cells in culture appear to be able to function in hypoxic conditions for relatively long periods of time compared with other cell types.

In our model, we have observed a correlation between the relative amount of processed laminin 5 γ2 chain and apoptosis. In HCE-T that were placed in a hypoxic environment immediately after trypsinization and replating, less γ2 chain is produced after 3 days in culture. However, the relative percentage of that γ2 chain that has been extracellularly processed is increased in hypoxic cells compared with normoxic cells. In an experiment not shown, HCE-T cultured for 5 days under normoxic conditions followed by 5 days under hypoxic conditions also deposited less total laminin 5 γ2 chain compared with cells cultured for the same amount of time in 20% oxygen only. However, a greater relative percentage of that γ2 chain is in the processed form in the cells exposed to hypoxic conditions.

It has been suggested that the acquisition of a motile phenotype can alleviate anoikis (Frisch and Francis, 1994). Extracellular processing of γ2 chain may be associated with the motility function of laminin 5. Taken together, between 1 and 5 days exposure to hypoxia, human corneal epithelial cells can effect an upregulation in the extracellular processing of laminin 5 γ2 chain in an attempt to adopt a motile phenotype and escape hypoxia-mediated apoptosis. However, the cells cannot sustain this response and at time points longer than 5 days in culture increased laminin 5 γ2 chain processing stops, and cells become apoptotic. Although the cells can upregulate processing of laminin 5 γ2 chain during short periods of hypoxic stress, the total amount of laminin 5 protein is consistently decreased. Based on our observations, we hypothesize that a decrease in total laminin 5 protein content, which in our model system occurs by 3 days in hypoxic culture, precedes the increased apoptosis (noted by mitochondrial function and PARP cleavage) observed by 7 days in culture. This suggests that the decreased laminin 5 present in the extracellular matrix of cultured human corneal epithelial cells contributes to initiation of hypoxia-mediated apoptosis.

The identity of the enzyme responsible for the extracellular processing of the laminin 5 γ2 chain is currently unknown. It has been suggested, though, that matrix metalloprotease 2 (MMP2) can cleave the 105 kDa γ2 chain into an 80 kDa γ2x species (Gianelli et al., 1999). This form of laminin 5 γ2 chain is correlated with a motile phenotype in breast tissue. To determine if these enzymes are involved in motile responses in corneal epithelium, we are currently determining the effect of hypoxia on MMP levels in HCE-T and HCEC.

Gonzales et al. (Gonzales et al., 1999) have shown that function-blocking antibodies to laminin 5 inhibit epithelial cell proliferation, implicating a cell signaling pathway involving laminin 5 (Gonzales et al., 1999). We show herein that hypoxia also inhibits epithelial cell proliferation. In addition, we show that hypoxia results in altered laminin 5 protein levels, and that loss of laminin 5 or function-blocking antibodies to laminin 5 upregulate apoptosis in cultured human corneal epithelial cells. The ability of cell-matrix interactions to generate cell survival signals, and the loss of those signals upon cell detachment from matrix, is the underlying mechanism of anchorage-dependent apoptosis (Meredith et al., 1993). Within corneal basement membrane, we propose that laminin 5 plays a bi-functional role in suppressing anchorage-dependent apoptosis: to ensure cell-matrix adhesion and to transmit extracellular survival signals to the nucleus. We hypothesize that one mechanism of hypoxia-mediated apoptosis in human corneal epithelium involves perturbation of the laminin 5-mediated cell signaling pathway proposed by Gonzales et al. (Gonzales et al., 1999).

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