Laminin-2 stimulates the proliferation of epithelial cells in a conjunctival epithelial cell line

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Abstract. Laminin-2 (LN-2, $\alpha 2\beta 1\gamma 1$) is a basement membrane-associated laminin isoform usually considered in the context of muscle and nerve tissues. To test the hypothesis that LN-2 can additionally modulate epithelial cell biology, an analysis of the role of LN-2 in cell adhesion, activation of signalling intermediates and proliferation was undertaken. A virally transformed human conjunctival epithelial cell line (HC0597) was utilized in this study. Adhesion assays using function-inhibiting antibodies demonstrated that $\alpha 3\beta 1$ integrin is essential for the rapid attachment of conjunctival epithelial cells to LN-2. Bromodeoxyuridine (BrdU) incorporation analyses revealed that, compared with LN-1 or LN-10, LN-2 significantly promotes epithelial proliferation. Phosphorylation of the signalling intermediates Erk1/2 and Akt-1 was observed within 15 min of cell adhesion to LN-2. Inhibiting $\alpha 3\beta 1$ integrin function decreased total cellular phosphotyrosine levels, specifically inhibited phosphorylation of Erk1/2 and Akt-1, and dampened the proliferation response of epithelial cells adherent to LN-2. Inhibition of Erk or Akt activation inhibited cell proliferation in a dose-dependent manner. However, the inhibition of Erk resulted in a stronger suppression of proliferation compared with Akt inhibition. From these results, it is concluded that human conjunctival epithelial cells adhere to immobilized LN-2 using $\alpha 3\beta 1$ integrin. $\alpha 3\beta 1$ integrin/LN-2 signalling, transduced primarily through an Erk pathway, enhances epithelial cell proliferation. These results demonstrate that LN-2 can impact on epithelial cell biology in addition to nerve and muscle, and provide information regarding the role of this isoform in ocular surface epithelial cells.

INTRODUCTION

Laminins are a family of extracellular matrix glycoproteins typically localized to basement membranes underlying epithelia, nerve and muscle. Laminin isoforms are crucial to cell-extracellular matrix integrity and, in general, play roles in epithelial proliferation, adhesion,

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migration, differentiation and survival (Ekblom *et al.* 1998; Colognato & Yurchenco 2000). Laminin-2 (LN-2, merosin) is composed of a heterotrimer of $\alpha 2\beta 1\gamma 1$ chains. LN-2 and LN-4 ($\alpha 2\beta 2\gamma 1$) are the most abundant isoforms in muscle and peripheral nerve basement membranes and are vital for normal functioning of these tissues (Sunada *et al.* 1994; Xu *et al.* 1994; Frost *et al.* 1996; Colognato *et al.* 1997; Kuang *et al.* 1998). For example LN-2 has been implicated in aspects of bronchial myogenesis by stimulation of peribronchial mesenchymal cell spreading and elongation (Relan *et al.* 1999).

The majority of studies regarding LN-2 have focused on localization and function in developing and adult muscle and peripheral nerve tissues. However, this isoform is present in other tissues as well (Vuolteenaho *et al.* 1994), including the basement membrane associated with epidermis (Schuler & Sorokin 1995; Miner *et al.* 1997; Qin *et al.* 1997) and intestine (Orian-Rousseau *et al.* 1996). LN-2 is also a component of the basement membrane associated with conjunctival epithelium of the ocular surface (Ljubimov *et al.* 1995; Touri *et al.* 1996; Qin *et al.* 1997; Ljubimov *et al.* 1998). With the exception of LN-2 localization in normal and damaged eye, essentially nothing is currently known regarding its function in ocular surface epithelium.

In a study assessing laminin α chain function Hoffman *et al.* (1998) demonstrated that a peptide fragment of the $\alpha 2$ chain stimulated multicellular morphological organization of human submandibular gland cells in suspension culture. The authors surmised that different laminin α chains might be involved in different morphological events during development. This notion is supported by our work showing that during embryonic development of the ocular surface of the mouse eye LN-1 ($\alpha 1\beta 1\gamma 1$) expression was ubiquitous. In contrast, expression of the laminin $\alpha 2$ chain was restricted to the conjunctival basement membrane (Qin *et al.* 1997). Expression of the laminin $\alpha 2$ chain was first detected during the same developmental period in which differentiated (keratin K4-positive) conjunctival epithelial cells were initially observed.

We hypothesized from our developmental work that laminin isoforms play vital roles in the regulation of ocular surface epithelial cell adhesion, survival and differentiation. To test the hypothesis, we demonstrated that the dynamics of tyrosine phosphorylation of signalling intermediates, including focal adhesion kinase (FAK), extracellular signal-related kinase (Erk-1) and Erk-2, were different in human conjunctival epithelial cells adherent to laminin-10 (LN-10, $\alpha 5\beta 1\gamma 1$) compared with laminin-1 (LN-1, $\alpha 1\beta 1\gamma 1$) (Lin *et al.* 2000). Additionally, we demonstrated that laminin signalling via Erk is mediated by integrins, cell surface heterodimers that function as receptors for extracellular matrix proteins (Hynes 1987; Yamada & Miyamoto 1995). Integrins can also activate the phosphatidylinositol 3-kinase (PI 3-kinase) signalling pathway leading to the activation of protein kinase B, also known as Akt (Rameh & Cantley 1999). Thus, in addition to Erk, Akt-mediated signalling can also play a role in transducing signals in response to integrin-induced epithelial cell adhesion to extracellular matrix (Khwaja *et al.* 1997).

We have extended our analyses of laminin isoform function in epithelial cells by determining the role of LN-2 in conjunctival epithelium. We hypothesize that LN-2, although usually considered a component of muscle and nerve basement membranes, can also impact on epithelial cell biology. In this report, we provide evidence that LN-2 plays roles in regulating conjunctival epithelial cell adhesion to extracellular matrix via $\alpha 3\beta 1$ integrin. We also provide evidence that LN-2 ligation to epithelial cells via $\alpha 3$ integrin activates both Erk1/2 and Akt-1 signalling intermediates. Studies using signalling intermediate blockers suggest that LN-2/ $\alpha 3\beta 1$ integrin modulates the proliferation of conjunctival epithelial cells primarily via Erk1/2.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies (mAb) directed against the integrin $\alpha 3$ chain (Clone P1B5) were purchased from Gibco-BRL Life Technologies (Gaithersburg, MD, USA). P1B5 mAb has been documented to inhibit $\alpha 3$ integrin function in cultured epithelial cells (Carter *et al.* 1990). mAb to integrin $\beta 1$ (Clone 6S6), integrin $\beta 4$ (Clone ASC-3) and integrin $\alpha 6$ (GoH3) subunits were purchased from Chemicon International (Temecula, CA, USA). GoH3 has been shown to block $\alpha 6$ integrin function in cultured epithelial cells (Sonnenberg *et al.* 1991). Polyclonal Ab against phosphorylated Akt-1 (pAkt-1), monoclonal Ab against phosphotyrosine residues (PY20) and polyclonal Ab against FAK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). mAb against diphosphorylated Erk1/2 (pErk1/2) was purchased from Sigma Chemical Co. (St Louis, MO, USA).

Cell culture

The human conjunctival epithelial cell line HC0597 was maintained in T-75 flasks in serum-free KGM, which consists of keratinocyte basal medium (KBM) supplemented with 0.1% bovine insulin, 0.1% human epidermal growth factor, 0.4% bovine pituitary extract and 0.1% hydrocortisone. Medium was replaced every other day and cells were trypsinized at approximately 80% confluence for use in experiments or for further passage (Lin *et al.* 2000). HC0597 were used between passage 12 and 25. HC0597 cells are viable to passage 30.

Cell adhesion assays

Adhesion assays were performed according to the procedure of Zhang & Kramer (1996) as described previously in our work (Kuropakus *et al.* 1999). Wells of a 96-well microtitre plate were coated with 1 μ g LN-2 (100 μ l of a 10- μ g/ml solution). Human LN-2 (Gibco-BRL) is purified from an EDTA/salt extract of placenta followed by size exclusion and ion exchange chromatography. The preparation contains LN-2 ($\alpha 2\beta 1\gamma 1$) and is devoid of LN-1. Trypsinized HC0597 cells were pelleted by centrifugation in KBM containing trypsin-neutralizing solution (Cascade Biologics, Portland, OR, USA). Cell pellets were then washed three times in KBM/0.1% bovine serum albumin (BSA) and resuspended in the same medium.

Trypsinized and washed cells were incubated in medium containing 25 μ g/ml P1B5 (anti- $\alpha 3$), GoH3 (anti- $\alpha 6$), 6S6 (anti- $\beta 1$) or ASC-3 (anti- $\beta 4$) for 30 min at 37 °C. This concentration of antibody has been determined to be maximally effective in studies not shown herein. As a control, cells were incubated in medium containing 25 μ g/ml non-immune IgG. Cells were plated at 2×10^4 cells/well on LN-2-coated microtitre plates and incubated for 1 h at 37 °C and 5% CO₂. The wells were washed with KBM/0.1% BSA to remove unattached cells. Attached cells were fixed, stained with 2% crystal violet blue and lysed in 2% sodium dodecyl sulfate (SDS). The absorbance of the lysate was measured at 570 nm wavelength (A₅₇₀) and absorbance of BSA controls subtracted. The adhesion assays were repeated three times, using 12 wells of each treatment and control each time. Data are represented as bar graphs of percentage cell adhesion compared with non-immune IgG control set to 100%. Errors are expressed as $\pm$ SEM.

In a separate analysis, wells of a 96-well microtitre plate were coated with 1 μ g LN-1, LN-2 or LN-10 (100 μ l of a 10- μ g/ml solution). EHS laminin, containing LN-1, was purchased from Sigma. A placental laminin preparation containing LN-10 was purchased from Gibco. The colourimetric cell adhesion assay, without the addition of antibodies, was completed and analysed as described in the previous paragraphs. These results are reported as mean A_{570nm}. Errors are expressed as $\pm$ SEM.

Cell proliferation assay: colourimetric BrdU incorporation

Wells of a 96-well microtitre plate were coated with 1 µg LN-1, LN-2 or LN-10. HC0597 cells were plated at 2×10^4 cells/well and incubated for 24 or 48 h. Four hours prior to analysis, 5-bromo-2'-deoxyuridine (BrdU) was added to each well to a final concentration of 10 µM. Cells were fixed and DNA denatured using reagents supplied with a cell proliferation assay kit (Roche Diagnostics, Indianapolis, IN, USA). Cells were incubated with anti-BrdU peroxidase conjugate, washed and incubated with colour development substrate. The absorbance of the samples was measured at 450 nm wavelength (A450). The assays were repeated three times, using 12 wells of each treatment for each assay. The results of the assay are represented as mean $A450 \pm$ SEM.

To analyse integrin effects on proliferation, cells were plated onto LN-2-coated wells and allowed to adhere for 4 h. The culture medium was removed and replaced with 100 µl medium containing 25 µg/ml P1B5, GoH3, 6S6 or ASC-3. Cells were incubated with the same volume of 25 µg/ml non-immune IgG as control. Cells were incubated for 24 or 48 h in the presence of the antibody. The proliferation assay was completed as described. The assays were repeated three times, using 12 wells of each treatment for each assay. The results are presented as bar graphs of relative percentage of cell proliferation compared with IgG control (set at 100%).

In separate analyses, cells were plated onto LN-2-coated wells and treated with 10, 50 or 100 µM PD98059 (Sigma) for 24 h prior to the completion of the cell proliferation assay (Ito *et al.* 1997). Cells were also treated with 1, 5 or 10 µM wortmannin (Gary & Mattson 2001). The assays were repeated three times, using 12 wells of each treatment for each assay. The results of these two proliferation assays are also represented as bar graphs of percentage cell proliferation compared with control cells treated with dimethylsulfoxide (DMSO) vehicle only set at 100%. Errors are expressed as SEM. Statistical analysis was completed using the two-tailed unpaired Student's t-test with $P \leq 0.05$.

Bright-field microscopy

Categories of some wells of the microtitre plate established for the 24 h cell proliferation assay using function-blocking integrin antibody were not processed. These cells were instead fixed in 3.7% formalin and stained with 1% toluidine blue. The cells were photographed using bright-field microscopy.

Western blot analysis of protein tyrosine phosphorylation

HC0597 cells were trypsinized, neutralized, pelleted and washed three times in 1 : 1 Dulbecco's modified Eagle's medium (DMEM)/0.1% bovine serum albumin (BSA), Medium F12 containing 20 µM sodium orthovanadate and 5 mM sodium fluoride. Cells were resuspended in the same medium and plated on to 60-mm tissue culture plates coated with 1 µg LN-2. After 5, 15, 30, 60, 90 or 120 min incubation, the plates were rinsed with phosphate-buffered saline (PBS) and the attached cells were lysed in buffer containing 10 mM Tris-HCl pH 6.8, 150 mM NaCl, 10% glycerol, 1% mercaptoethanol, 2% SDS, 200 µM sodium orthovanadate and 5 mM sodium fluoride (Lin *et al.* 2000). Suspended cells at time 0 were used as control and were pelleted and lysed in the same buffer.

Cell lysates were analysed for total protein using the method of Henkel & Beiger (1994). Samples containing 20 µg total protein were separated by 7.5% SDS-PAGE and transferred to nitrocellulose membrane for western blot analysis. Molecular weight standards were run with the gels. The blots were blocked in Tris-buffered saline (TBS) containing 1% milk and then incubated in antibody to pTyr, FAK, pErk1/2 or pAkt-1 diluted in TBS containing 0.1% Tween-20 and 0.2% milk. Proteins containing phosphorylated residues were detected as exposed bands on X-ray film using alkaline phosphatase-conjugated goat secondary antibody coupled with

the Immune-Star chemiluminescence detection system (Bio-Rad Laboratories, Hercules, CA, USA).

In a separate experiment, HC0597 cells were treated with 25 µg/ml mAb P1B5, 6S6 or non-immune IgG for 30 min at 37 °C in a tissue culture incubator. Cells were plated on to 60-mm tissue culture plates coated with 1 µg LN-2 and incubated for 120 min. Cells were then processed for western blot analysis as described. For a cohort of analyses the exposed X-ray films resulting from chemiluminescence detection were scanned and the density of immunoreactive protein bands were measured in arbitrary units of density per µg total protein using ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA, USA).

Cellular phosphotyrosine ELISA

Wells of a 96-well microtitre plate were coated with 1 µg LN-1, LN-2 or LN-10 and blocked with BSA. HC0597 cells were plated at 3×10^4 cells/well in KBM/0.1% BSA and allowed to adhere for 4 h. Non-adherent cells were removed by washing the wells with PBS prior to completion of the cellular phosphotyrosine ELISA (Upstate Biotechnology, Lake Placid, NY, USA). Adherent cells were fixed in methanol : acetic acid (50 : 50). Washed cells were then incubated with 50 µl of a 10-µg/ml solution of horseradish peroxidase-conjugated anti-phosphotyrosine antibody for 1 h. Washed cells were incubated with tetramethylbenzidine detection reagent for 30 min. Following acidification of the wells, the absorbance was measured at 450 nm (A450). Twenty-four wells of each treatment were measured and the data are represented as mean A450. Errors are expressed as SEM.

In a second analysis HC0597 cells were plated at 3×10^4 cells/well in KBM/0.1% BSA and allowed to adhere for 4 h to LN-2. Cells were then treated with fresh medium containing 25 µg/ml P1B5, 6S6 or non-immune IgG as control. Cells were incubated for 2 h in the presence of the antibody and the assay was completed as described. Data are represented as bar graphs of per cent cellular phosphotyrosine compared with IgG controls set to 100%. Errors are expressed as SEM.

RESULTS

Epithelial cell adhesion to LN-2 is mediated by $\alpha 3$ integrin

Integrins reported to be involved in ocular surface epithelial cell attachment to laminins are $\alpha 3\beta 1$ and $\alpha 6\beta 4$ (Filenius *et al.* 2001). To identify integrins mediating conjunctival epithelial cell adhesion to LN-2, HC0597 cells were incubated with function-blocking mAb to $\alpha 3$ (P1B5), $\alpha 6$ (GoH3), $\beta 1$ (6S6) or $\beta 4$ (ASC-3) integrin subunits prior to performing cell adhesion assays (Fig. 1). Disrupting the function of $\alpha 6$ integrin partially inhibited rapid adhesion to immobilized LN-2 by less than 10%, while inhibiting $\beta 4$ integrin had no deleterious effect on adhesion. In contrast, blocking the function of $\alpha 3$ integrin inhibited adhesion to LN-2 by 55%. Inhibiting $\beta 1$ integrin produced a significant modulation of attachment and blocked rapid adhesion by 79%. Cell adhesion assays utilizing function-inhibiting antibody suggest that in conjunctival epithelial cells $\alpha 3\beta 1$ integrin is utilized in attachment mechanisms to LN-2.

Erk1/2 and Akt-1 phosphorylation is increased subsequent to epithelial cell adhesion to LN-2

It is well recognized that subsequent to epithelial cell adhesion to extracellular matrix an up-regulation in the phosphophorylation of signalling pathway intermediates is observed. To

Figure 1. Effect of integrin inhibition on human conjunctival epithelial cell adhesion to immobilized LN-2. HC0597 were allowed to adhere to wells of a microtitre plate coated with LN-2 in the presence of mAb to α 3 integrin (P1B5), α 6 integrin (GoH3), β 1 integrin (6S6) or β 4 integrin (ASC-3) prior to a colourimetric cell adhesion assay. Results are expressed as the percentage of cell adhesion compared with non-immune IgG control set to 100. Each bar of the graph represents the mean percentage $\pm$ SEM of three replicate experiments.

determine how laminin isoforms affect this, we measured the phosphorylation response of HC0597 cells adherent to LN-1, LN-2 or LN-10 using an ELISA that measures total cellular phosphotyrosine content (Fig. 2a). Compared with BSA control, cell adhesion to LN-2 resulted in a significant increase in phosphorylation ($P < 0.0001$). LN-2 additionally increased total cell phosphorylation compared with LN-1 ($P < 0.0001$), however, no difference was observed compared with cells adherent to LN-10. The ELISA was then used to provide information regarding the effect of blocking α 3 β 1 integrin function on the global protein phosphorylation response in epithelial cells subsequent to adherence to LN-2 (Fig. 2b). When compared with isotype-matched Ab control, inhibiting the ligation of α 3 or β 1 integrin to LN-2 in HC0597 cells significantly decreased total cellular phosphotyrosine levels ($P < 0.0001$).

The cellular phosphotyrosine ELISA does not provide for the analysis of specific signalling pathways. To identify the signalling pathway intermediates that are phosphorylated in response to epithelial cell adhesion to LN-2, western blot analysis of HC0597 cells was performed. Antibody specific for phosphorylated forms of Erk1/2 and Akt-1 were used to assay for activation of intermediates involved in Erk and PI-3 kinase signalling pathways, respectively. The phosphorylation of both Erk-1/2 and Akt-1 was increased over time 0 control levels at 30 min of adhesion to LN-2 (Fig. 3). Enhanced phosphorylation was sustained over the assay period of 120 min.

We have previously demonstrated differences in FAK activation in HC0597 cells adherent to LN-1 or LN-10 (Lin *et al.* 2000). To extend this previous analysis, we determined tyrosine phosphorylation kinetics of FAK in HC0597 cells adherent to LN-2 (Fig. 4). FAK phosphorylation continued to increase over time 0 control levels for the duration of the assay. This contrasts to a previous observation for HC0597 cells adherent to LN-10 (Lin *et al.* 2000), which showed a peak in phosphorylation at 30 min.

The cellular phosphotyrosine assay provided information regarding the general response of HC0597 adhesion to LN-2, and suggested that α 3 integrin plays a major role in transducing LN-2-mediated signals. To determine the effect of blocking α 3 integrin function on the phosphorylation of Erk and PI-3 kinase pathway signalling intermediates, western blot analysis of phosphoErk-1/2 and phosphoAkt-1 was completed (Fig. 5). Semi-quantitative densitometric analysis showed that, compared with antibody control, treating HC0597 cells with function-inhibiting antibody to β 1 integrin resulted in a 5-fold decrease in phosphoErk-1 expression and a 2.8-fold

Figure 2. (a) Effect of laminin isoforms on cellular phosphotyrosine levels in human conjunctival epithelial cells. HC0597 cells were cultured on immobilized LN-1, LN-2 or LN-10, and total cellular phosphotyrosine levels were assayed. Levels are significantly increased in cells adherent to LN-2 compared with BSA control or to LN-1 (star indicates $P < 0.0001$ for both comparisons). **(b) Effect of integrin inhibition on cellular phosphotyrosine levels in human conjunctival epithelial cells.** HC0597 cells were cultured on immobilized LN-2 in the presence of mAb to α 3 integrin (P1B5) or β 1 integrin (6S6). HC0597 cultured in the presence of non-immune IgG was used as control. After the culture period, total cellular phosphotyrosine levels were assayed. Results of the assay are expressed as per cent cellular phosphotyrosine levels compared with control set at 100%. Each bar of the graph represents the mean percentage $\pm$ SEM. Antibody treatment results in a significant decrease in phosphorylation compared with control (stars indicate $P < 0.0001$).

Figure 3. Tyrosine phosphorylation of signalling intermediates in human conjunctival epithelial cells adherent to immobilized LN-2. HC0597 cells were incubated on LN-2 for the times indicated. Cell lysates containing equal total protein were resolved by 7.5% SDS-PAGE and subjected to western blot analysis to determine relative expression of phosphorylated Erk-1 and Erk-2 (pErk-1, pErk-2) and phosphorylated Akt-1 (pAkt-1).

decrease in phosphoAkt-1 expression. Treating HC0597 cells with function-inhibiting antibody to α 3 integrin resulted in a 2.5-fold relative decrease in phosphoErk-1 levels and a 1.3-fold relative decrease in phosphoAkt-1 levels.

Epithelial cell adhesion to LN-2 promotes proliferation via α 3 β 1 integrin

In addition to adhesion and migration, extracellular matrix proteins can regulate epithelial proliferation. Accordingly, a BrdU incorporation ELISA was utilized to test the effect of exogenous

Figure 4. Tyrosine-phosphorylation kinetics of FAK in human conjunctival epithelial cells. Tyrosine-phosphorylated FAK was identified using western blot analysis (blot not shown). The relative levels of tyrosine phosphorylation as a function of time of adherence to LN-2 is represented in arbitrary units as the density of the immunoreactive protein bands measured from scanned X-ray films divided by the total μ g of protein loaded on the gel. Error bars are not shown because the graph is representative of three analyses.

Figure 5. Effect of integrin inhibition on phosphorylation of signalling intermediates. HC0597 cells were incubated on tissue culture plastic coated with LN-2 for 2 h in the presence of control IgG or antibody to $\alpha 3$ or $\beta 1$ integrin. Cell lysates containing equal total protein were resolved by 7.5% SDS-PAGE and subjected to western blot analysis using antibody to phosphorylated Erk-1 and Erk-2 (pErk-1, pErk-2) and phosphorylated Akt-1 (pAkt-1).

LN-2 on conjunctival epithelial cell proliferation (Fig. 6). After 24 or 48 h in culture, HC0597 cells adherent to LN-2 showed a significant increase in BrdU incorporation compared with LN-10 ($P = 0.0271$ for 24 h; $P = 0.0008$ for 48 h) or to LN-1 ($P = 0.0058$ for 24 h; $P = 0.0006$ for 48 h). Cell proliferation was also significantly increased compared with bovine serum albumin substrate (data not shown).

Because proliferation results can be skewed by differences in the total number of cells analysed, we determined the adhesion characteristics of the conjunctival cells to each of the LN isoforms (Fig. 7). Compared with controls, LN-2 significantly enhanced the rapid adhesion of HC0597 cells ($P < 0.0001$). In contrast, adhesion was inhibited compared with LN-1 or LN-10. The inverse observations of relatively decreased adhesion, but relatively enhanced proliferation, suggests that the proliferation effects of LN-2 are not due solely to differences in the total number of cells adherent to the assay substrate.

We next used the BrdU incorporation ELISA to examine the function of LN-2/integrin ligation in epithelial proliferation. The cell adhesion assay shown in Fig. 2 demonstrated that treating cells with Ab to $\alpha 3$ or $\beta 1$ integrins inhibited the rapid attachment of HC0597 cells to LN-2. It could be argued then that antibody treatment may non-specifically inhibit proliferation simply because adhesion is inhibited. To circumvent this potential experimental artifact,

Figure 6. Effect of laminin isoforms on human conjunctival epithelial cell proliferation. HC0597 cells were allowed to adhere to laminin-10 (LN-10), laminin-1 (LN-1) or laminin-2 (LN-2) for 24 or 48 h prior to BrdU incorporation assays. Proliferation is represented as $A_{450nm} \pm SEM$. Cells adherent to LN-2 demonstrated significantly enhanced proliferation compared with LN-10 (star indicates $P = 0.0271$ for 24 h; $P = 0.0008$ for 48 h) or to LN-1 (star indicates $P = 0.0058$ for 24 h; $P = 0.0006$ for 48 h).

Figure 7. Effect of laminin isoforms on human conjunctival epithelial cell adhesion. HC0597 were allowed to adhere to wells of a microtitre plate coated with LN-1, LN-2 or LN-10 prior to a colourimetric cell adhesion assay. Each bar of the graph represents the mean $A_{570nm} \pm SEM$ of three replicate experiments. Adhesion to LN-2 is significantly enhanced compared with BSA (star indicates $P < 0.0001$).

HC0597 cells were permitted to adhere and spread on immobilized LN-2 for a short period prior to treatment with function inhibiting Ab to $\alpha 3$, $\alpha 6$, $\beta 1$ or $\beta 4$ integrin subunits. This approach would normalize for the number of cells attached to LN-2 prior to experimental manipulation. Compared with the antibody control, blocking $\alpha 6$ or $\beta 4$ integrin ligation to LN-2 had no or minimal inhibitory effect on epithelial cell proliferation over 24 or 48 h (Fig. 8). In contrast, blocking the function of $\alpha 3$ or $\beta 1$ integrin resulted in 20–30% inhibition of BrdU incorporation.

Figure 8. Effect of integrin inhibition on proliferation of human conjunctival epithelial cells cultured on immobilized LN-2. HC0597 cells were cultured for 24 h or 48 h on LN-2 in the presence of mAb to α 3 integrin (P1B5, black bars), α 6 integrin (GoH3, vertically striped bars), β 1 integrin (6S6, horizontally striped bars) or β 4 integrin (ASC-3, hatched bars). HC0597 cultured in the presence of non-immune IgG was used as control (open bars). After the culture period, cell proliferation was assayed by measuring BrdU incorporation. Results of the assay are expressed as per cent proliferation compared with control set at 100%. Each bar of the graph represents the mean percentage $\pm$ SEM of three replicate experiments.

Figure 9. Effect of integrin inhibition on spreading of human conjunctival epithelial cells cultured on immobilized LN-2. HC0597 cells were cultured for 24 h in the presence of non-immune IgG as control (a), or in the presence of mAb to α 3 integrin (b) or α 6 integrin (c). Note that the morphology or spreading of antibody-treated cells does not differ from controls.

As evidence that the inhibition of proliferation by α 3 and β 1 integrin Ab was not a consequence of altered cell-extracellular matrix attachment or aberrations in cell morphology, some of the HC0597 cells contained within the microtitre plate used for the BrdU incorporation assay were instead fixed and stained for light microscopy. The presence of either α 3 or β 1 integrin subunit Ab had minimal deleterious effects on cells that adhered to and spread on the LN-2 matrix (Fig. 9). The morphology of control cells (Fig. 9a) is indistinguishable from that of antibody-treated cells (Fig. 9b and c). Additionally, there were no adverse affects on cell viability (data not shown).

Erk1/2 and Akt-1 modulate conjunctival epithelial cell proliferation

The inhibition of Erk1/2 phosphorylation in cells treated with function-inhibiting α 3 integrin antibody suggests that this integrin can mediate Erk1/2 signalling pathways initiated by cell adhesion to LN-2. We have also demonstrated that α 3 integrin interaction with LN-2 is vital for cell proliferation. To link Erk-1/2 phosphorylation and activation to regulation of conjunctival epithelial cell proliferation, HC0597 cells were treated with PD98059 and assayed for BrdU incorporation. PD98059 has been shown to specifically inhibit the Erk1/2 MAPkinase signalling pathway (Alessi *et al.* 1995). Treatment of HC0597 cells with PD98059 resulted in a decrease in BrdU incorporation compared with vehicle-treated controls (Fig. 10). The effect of the

Figure 10. Effect of PD98059 on proliferation of human conjunctival epithelial cells cultured on immobilized LN-2. HC0597 cells were cultured for 24 h in the presence of varying concentrations of PD98059. HC0597 cells cultured in the absence of the inhibitor (0, vehicle only) were used as control. After the culture period, cell proliferation was assayed by measuring BrdU incorporation. Results of the assay are expressed as per cent proliferation compared with control set at 100%. Each bar of the graph represents the mean percentage $\pm$ SEM of three replicate experiments. At 50 and 100 μ M PD98059 the inhibition of proliferation was significant compared with control (stars indicate $P < 0.0001$).

Figure 11. Effect of wortmannin on proliferation of human conjunctival epithelial cells cultured on immobilized LN-2. HC0597 cells were cultured for 24 h in the presence of varying concentrations of wortmannin. HC0597 cells cultured in the absence of the inhibitor (0, vehicle only) were used as control. After the culture period, cell proliferation was assayed by measuring BrdU incorporation. Results of the assay are expressed as per cent proliferation compared with control set at 100%. Each bar of the graph represents the mean percentage $\pm$ SEM of three replicate experiments. At 10 μ M wortmannin proliferation was significantly decreased compared with vehicle control (star indicates $P = 0.0022$).

inhibitor on cell proliferation was dose-dependent, and at 50 and 100 μ M PD98059 inhibition was significant compared with control ($P < 0.0001$).

Figure 4 revealed that Akt-1 was also phosphorylated following HC0597 cell adhesion to LN-2. HC0597 cells thus were treated with wortmannin, an inhibitor of PI 3-kinase and the downstream signalling intermediate Akt-1 (Gary & Mattson 2001). The cells were subsequently assayed for BrdU incorporation. Similar to PD98059, treatment of cells with wortmannin resulted in a dose-dependent decrease in proliferation compared with vehicle control (Fig. 11). At 10 μ M wortmannin proliferation was significantly decreased compared with vehicle control ($P = 0.0022$). Although inhibition was significant, when compared with PD98089, treatment of HC0597 cells with wortmannin was relatively less detrimental with respect to proliferation.

DISCUSSION

We have used a virally transformed human conjunctival epithelial cell line, HC0597, in our studies (Lin *et al.* 2000; Lin & Kurpakus-Wheater 2002; Sosne *et al.* 2002). The applicability of results gained from using a transformed cell line to the *in vivo* state can be a concern. However, HC0597 cells possess two essential characteristics of *in vivo* conjunctival epithelium; they express the differentiation marker keratin K4 (Lin *et al.* 2000) and they secrete LN-2 into the extracellular matrix (data not shown). Expression of keratin K4 and LN-2 by HC0597 cells *in vitro* support the use of the cell line as a model relevant to conjunctival epithelial cell responses to laminin in the intact eye.

LN-2 is localized to the conjunctival basement membrane of the ocular surface (Ljubimov *et al.* 1995; Touri *et al.* 1996; Qin *et al.* 1997; Ljubimov *et al.* 1998). However, it is not present in normal corneal basement membrane, suggesting that this isoform may contribute to tissue-specific functions in ocular surface epithelium. To identify general functions of LN-2 in epithelial cells, and specific functions of LN-2 in conjunctival epithelium, we assayed for its ability to affect adhesion, motility, signalling, and proliferation in cultured HC0597 cells.

Herein, we show that $\alpha 3\beta 1$ integrin is a conjunctival epithelial cell receptor for LN-2. In HT1080 human fibrosarcoma cells, antibody to $\alpha 3$ integrin blocks adhesion to LN-2 by approximately 25% (Colognato *et al.* 1997). Antibodies to $\beta 1$ integrin have been shown to dramatically inhibit adhesion of normal human renal cortex epithelial cells and renal carcinoma cells to LN-2 (Jenq *et al.* 1993). These observations by other investigators, in two diverse human cell types, support our work suggesting that $\alpha 3\beta 1$ integrin is a functional receptor for LN-2 in tissues other than nerve or muscle. Subsequent to conjunctival epithelial adhesion to LN-2 $\alpha 3\beta 1$ integrin ligation results in enhanced cell proliferation. Circumstantial evidence of a role for LN-2 in proliferation is given by the localization of the $\alpha 2$ chain of LN-2 to small intestinal crypts, the site of stem cells (Beaulieu & Vachon 1994; Leivo *et al.* 1996; Bouatrouss *et al.* 2000). Additionally, the *in vitro* survival of thymocytes from normal mice was maintained by LN-2 (Iwao *et al.* 2000).

We suggest that the proliferative response of human conjunctival epithelial cells adherent to LN-2 is mediated primarily via Erk1/2 signalling. In support of this we show that inhibiting the $\alpha 3$ or $\beta 1$ integrin subunit function dampens the activation of Erk1/2 to a greater degree than Akt-1. Additionally, inactivation of Erk1/2 results in a greater suppression of cell proliferation compared with inactivation of Akt-1. We used doses of PD98059 and wortmannin within ranges reported by other investigators (Ito *et al.* 1997; Gary & Mattson 2001), thus we do not believe that our observations are due to concentration differences. Both the Erk and Akt pathways have been found to enhance cell survival, growth and proliferation (Khawaja & Downward 1997; Yujiri *et al.* 1998; Erhardt *et al.* 1999; Jasleen *et al.* 2000; Wilkinson & Millar 2000). However, in human gingival fibroblasts adherent to cementum-derived attachment protein, cell proliferation is not affected by PD98059, the Erk inhibitor (Komaki *et al.* 2000). Gu *et al.* (2002) showed that in adenocarcinoma, survival signals from LN-10 are PI 3-kinase/Akt mediated, but Erk signalling was operative in carcinoma cells adherent to fibronectin.

Conflicting reports of the role of signalling molecules in proliferation may be explained by the notion of cross-talk (Moelling *et al.* 2002). Cross-talk between the Ras-Raf-MEK-Erk and PI 3-kinase/Akt pathways has been demonstrated in several cell systems. For example Rommel *et al.* (1999) reported that inhibition of Erk or activation of Akt pathways promoted myotube formation. However, Laprise *et al.* (2002) suggested that, although myoblast survival is dependent on Erk pathways, the PI 3-kinase pathway is not involved in myoblast or myotube survival. It

has been hypothesized that Ras-Raf-MEK-Erk/Akt cross-talk may be regulated by ligand type or concentration, cellular background or stage of differentiation (Moelling *et al.* 2002). Correspondingly, ligand type and ligand concentration may contribute to cellular responses to proliferation or differentiation. The role of ligand type, for instance, may explain why we hypothesize that Erk plays a major role in mediating LN-2 responses, while Gu *et al.* (2002) suggests that signals from LN-10 are PI 3-kinase/Akt mediated.

An alternate explanation for the co-activation of Erk and Akt pathways may reside in the motif structure of the LN-2 molecule. The $\alpha 2$ chain of LN-2 contains three epidermal growth factor (EGF)-like repeats (Bernier *et al.* 1995). It has been suggested that in addition to integrin engagement, Akt may play a role in transducing survival signals in response to growth factors (Khawaja *et al.* 1997; Downward 1998). It is possible that $\alpha 3\beta 1$ integrin is involved in activation of Erk, while the EGF-like repeats of laminin $\alpha 2$ chain are initiating PI3-kinase/Akt signalling via a growth-factor like mechanism. Alternately, the possibility of other $\beta 1$ integrin heterodimers in the activation of signalling pathways should be considered. Although we have ruled out a role for $\alpha 6$ integrin in conjunctival epithelial cells, other subunits such as $\alpha 2$ are candidates. To this end, we are currently investigating the role of $\alpha 2\beta 1$ in laminin-mediated signalling.

In summary, we show in this study that human conjunctival epithelial cells adhere to LN-2 using $\alpha 3\beta 1$ integrin. $\alpha 3\beta 1$ integrin/LN-2 signalling, transduced primarily through an Erk pathway, enhances conjunctival epithelial cell proliferation.

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