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Effect of Mechanical Strain on Human Limbal Epithelial Cells \textit{In Vitro}

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\textbf{ABSTRACT} \textit{Purpose:} To investigate the effects of the mechanical cyclic strain on the extracellular matrix (ECM) production by cultivated human limbal epithelial cells (HLECs) \textit{in vitro}. \textit{Methods:} HLECs were repetitively stretched and relaxed by 20\% of their original length. Morphology of HLECs was observed, and concentrations of fibronectin and laminin V were measured. \textit{Results:} The strained HLECs were elongated and aligned perpendicular to the direction of force. Fibronectin and laminin V were highly expressed in the strain group compared with the control. \textit{Conclusions:} Cyclic strain induces the synthesis of fibronectin and laminin V in cultivated HLECs.

\textbf{KEYWORDS} cyclic strain; fibronectin; human limbal epithelial cell; laminin; mechanical strain

\textbf{INTRODUCTION}  
Cultivated limbal epithelium must continuously bear the mechanical stress caused by up-and-down movements of the eyelids after transplantation in human patients. These movements may interfere with adhesion of transplanted limbal epithelium; this is because complete adhesion complex formation is not achieved in cultured limbal epithelium on amniotic membranes even after 4 weeks of cultivation.\textsuperscript{1}

Mechanical strain has been shown to profoundly influence the proliferation, phenotype, function, and protein synthesis of a variety of cells such as keratinocytes,\textsuperscript{2} vascular smooth muscle cells,\textsuperscript{3} and intestinal epithelial cells\textsuperscript{4} \textit{in vitro}. Studies have shown an increase in extracellular matrix (ECM) synthesis in response to mechanical strain using human vascular smooth muscle cells\textsuperscript{4} or glomerular mesangial cells\textsuperscript{5} \textit{in vitro}. However, investigations of the production of ECM proteins, in corneal epithelial cells under mechanical strain, have not been reported to date.

We speculated that prior culture conditions with mechanical strain during cultivation might increase the capability of limbal epithelial cells to adhere to transplanted eyes with the usual continuous mechanical stress of lids. Therefore, in this study we evaluated the effect of mechanical cyclic strain on cultivated human limbal epithelial cells (HLECs) \textit{in vitro}. 

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MATERIALS AND METHODS

Cells and Culture Conditions

Human corneoscleral rims at 5 days after harvest were obtained from the Northwest Lions Eye Bank (Seattle, WA, USA) within 8 hr after penetrating keratoplasty. Each tissue was treated with 0.05% trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA) at 37°C, and then cells were collected every 20 min four times. NIH/3T3 (ATCC, Manassas, VA, USA) was used for a feeder layer and was treated with 4 μg/ml of mitomycin C (Sigma, St. Louis, MO, USA) at 37°C for 2 hr and next was plated to 80% confluence for coculture with epithelial cells. The suspended cells were seeded in 1.5 × 10^4 cells/cm² onto the 3T3-pretreated plates; primary culture with supplemented hormonal epithelial medium (SHEM) at 37°C in a carbon dioxide air incubator was developed over 2 weeks. The SHEM was a mixture of Dulbecco’s modified Eagle medium (DMEM; Cambrex, Baltimore, MO, USA) and Ham’s F12 medium (1:1 mixture), and included fetal bovine serum (FBS) (10%), insulin (5 mg/ml), cholera toxin (0.1 nmol/L), epidermal growth factor (10 ng/ml), and penicillin-streptomycin (PS) (50 IU/ml).

Cyclic Mechanical Strain

HLECs were stretched with the use of the Flexercell Stress Unit (FX4000, Flexcell International Corp., Hillsborough, NC, USA; kindly provided by Son YS) (Fig. 1). The cells were seeded at 3.9 × 10^5 cells/well onto the specialized 6 well plates that contained an elastomer flexible base (Bio Flex, NC, USA) and were incubated at 37°C, 5% CO₂ for 24 hr. These plates were precoated with 1.5 ml of mixed solution with collagen type I (1 ml) and sterile 75% ethyl alcohol (35 ml) and were air-dried at 25°C overnight. The plates were fixed into a mainfold that was placed in a CO₂ incubator, and a computer-controlled vacuum was applied to the mainfold, which stretched the flexible base of the culture plates and thereby stretched the adherent cells. In these experiments, the cells were cyclically stretched to 20% of their original length for 48 hr with a stretch: relaxation cycle at a rate of 0.5 Hz. The cells in the control group were cultured on identical plates in the same incubator in the absence of applied strain.

Western Blotting for Fibronectin and Laminin V

Western blot analysis was performed to analyze the content of fibronectin and laminin Vγ2 in the supernatant and pellet of the strain group and control group. The Western blot was made triplicate, and eight samples were used to evaluate the expression of fibronectin and laminin, respectively. After the collagen on the bottom of the plates was digested with 0.1% collagenase, cells were lysed with Laemmli lysis buffer including 2.4 M glycerol, 0.14 M Tris buffer (pH 6.8), 0.21 M sodium dodecyl sulfate (SDS), 0.3 mM bromophenol blue, and then boiled for 10 min. Samples were diluted with Laemmli lysis buffer containing 1.28 M β-mercaptoethanol. Ten micrograms per well of the sample were loaded on 6 ~ 8% SDS-polyacrylamide gels and transferred to a polyvinylidenedifluoride (PVDF) membrane (Lmmobilon-P, Millipore, Billerica, MA, USA). The membrane was incubated for 2 hr with rabbit anti-human fibronectin polyclonal antibody (1:1000, Sigma, St. Louis, MO, USA) and mouse anti-human laminin Vγ2 monoclonal antibody (1:250, Chemicon, Temecula, CA, USA), and for 1 hr with secondary antibodies (horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG). Immunoreactive protein was detected by chemiluminescence (Amersham, Arlington Heights, IL, USA).

Enzyme-Linked Immunosorbent Assay

To analyze the relative concentrations of fibronectin and laminin V in the cell supernatant and pellet of the strain and control groups, ELISA was performed. The ELISA was made triplicate, and eight samples were used to evaluate the expression of fibronectin and laminin, respectively. Samples (10 μg/well in 96-well plate) were coated with 50 mM carbonate/bicarbonate buffer (pH 9.6) and incubated with anti-laminin V,
On Western blotting, fibronectin was more highly expressed in the cell supernatant of the strained limbal epithelial cells compared with that of the control (A, B), and the expression of laminin V was significantly higher in the pellet of the strain group than that of the control (C, D). 1, Unstrained supernatant; 2, strained supernatant; 3, unstrained pellet; 4, strained pellet.

Immunostaining
To confirm that the cells, before and after cyclic strain, are limbal epithelial cells, we performed immunofluorescent staining for p63 (Chemicon) as a positive stem-cell marker and connexin 43 (Chemicon) as a negative stem-cell marker. The expression for fibronectin and laminin V was assessed by immunocytochemistry to know where the ECM proteins were deposited.

Statistical Analysis
Statistical analysis was performed using Sigmaster version 1.0 SPSS software (Chicago, IL, USA). A non-parametric Mann-Whitney U test was used to evaluate the level of significance of differences between groups. A p < 0.05 was considered significant.

RESULTS
Effect of Mechanical Strain on Cell Morphology
HLECs in the strain group were elongated and aligned perpendicular to the direction of force compared with those in the static group. These changes in morphology and alignment were most prominent in the periphery of the culture well.
Effect of Mechanical Strain on ECM Protein Production

HLECs subjected to cyclic strain were observed to more strongly express ECM proteins compared with the unstrained cells. Fibronectin was more highly expressed in the cell supernatant of the strain group compared with that of the control (p = 0.040, Mann-Whitney test) (Figs. 2A and 2B), while the expression of 105 KDa of laminin Vγ2 was significantly higher in the pellet of the strain group compared with the static group (p = 0.001, Mann-Whitney test) (Figs. 2C and 2D). As measured with ELISA, fibronectin was 2.55 ± 0.14 AU in the supernatant of the strain group and 2.35 ± 0.09 AU in the supernatant of the control group (p = 0.034, Mann-Whitney test) (Fig. 3A). Laminin Vγ2 was 1.48 ± 0.16 AU and 1.18 ± 0.16AU in the pellet of strain group and control, respectively (p < 0.01, Mann-Whitney test) (Fig. 3B). On immunocytochemistry, fibronectin and laminin V were markedly deposited in the periphery of the culture well compared with the center (Fig. 4A).

P63 was positive (Figs. 4B and 4C) and connexin 43 was negative in both strain and static groups. No differences in the marker expression were observed depending on the location of the cells in the culture plate.

DISCUSSION

Cultivated HLECs are exposed to the continuous strain forces of the eyelids after they are transplanted in the cornea, suggesting that epithelial-stromal interactions are important for the long-term survival of cultivated epithelium when grafted. Although many reports have presented cultivated epithelial cells that were successfully transplanted, fully differentiated epithelial sheets including well-developed adhesion complex requires relatively long-term culture.

Previous reports suggest that adaptive cellular responses to physical stimuli contribute to strain resistance; these responses include alterations in the cytoskeleton, integrin expression, and ECM protein synthesis in a variety of cells such as keratinocytes, vascular smooth muscle cells, osteoblasts, intestinal epithelial cells. However, studies evaluating the influence of mechanical stimuli on corneal limbal epithelial cells have not been reported to date. Therefore, we studied the effect of prior mechanical training on alteration of ECM production in corneal limbal epithelial cells and the effect on the epithelial adhesion in a transplanted sheet.

In our study, HLECs in response to strain elongated and aligned perpendicular to the force vector. These events occurred mainly in the periphery of the culture well, which was directly attributable to the heterogeneous nature of the strain on the flexible membrane; because the well periphery is a high-strain area as opposed to the low-strain center of the well. According to previous experiments using endothelial cells, there is a 24% strain at the periphery versus less than 1% in the center of the flexible well, if the membrane is subjected to 150 mmHg vacuum.

With regard to ECM production, several studies have shown increased matrix synthesis in response to mechanical strain using vascular smooth muscle cells, mesenchymal progenitor cells, keratinocytes, and glomerular mesangial cells in vitro or perfused rabbit aorta ex vivo. In the current study, mechanically
stimulated limbal epithelial cells produced more fibronectin and laminin V than the static control cells. Fibronectin is a multifunctional ECM glycoprotein and is present in the basement membrane zone of the cornea, limbus, and conjunctiva. It acts as an important adhesive factor because it is a ligand for integrin, which mediates binding of cells to the underlying matrix. HLECs are known to produce isoforms of fibronectin especially during cell adhesion to the substrata. Laminin V, as a major component of corneal basement membrane, also has a crucial role in corneal epithelial cell adhesion. However, we found the 105-kDa γ2 chain, which is a mature form, increased more markedly than the 150-kDa γ2 chain of laminin V, a precursor form, after cyclic straining. It should be noted that the proteolytic processing of the γ2 and α3 chains affects laminin V activity. The cleavage of the 150-kDa γ2 chain to the 105-kDa form is known to decrease the cell adhesion activity of laminin V but increases its cell motility activity. The induction of the synthesis of fibronectin could be enhanced rapid adhesion of the cyclic strained cells. The observation that cleavage of laminin V showed increase of the 105-kDa γ2 chain, in the strained epithelial cells, suggests that the cyclic strain can lead to rapid migration of the limbal epithelial cells during cultivation. Rapid migration and firm adhesion would be helpful to enhance the differentiation of the cultivated cells. These ECM proteins were markedly deposited in the periphery of the culture well compared with the center. It is consistent with the fact that the strain is stronger in the periphery, which is due to the mechanical characteristics of the flexible membrane.

Several mechanisms have been proposed to explain how cells recognize and respond to mechanical forces. The tensegrity model proposed by Ingber suggests that applied forces are transmitted directly to the nucleus through a direct connection from integrins through the cytoskeleton. The signal transduction model suggests that chemical mediators such as TGF-β1 or kinases such as protein kinase C are activated by integrins in focal adhesion in response to applied forces, leading to a signaling pathway eventually resulting in regulation of nuclear transcription and cell-cycle progression.

There are several limitations in our study. First, time-dependent changes in ECM protein production by strained HLECs were not evaluated. We selected 48 hr as the duration of strain, because we thought 48 hr
would be the most appropriate both to induce a significant ECM change and to reduce the risk of losing the limbal cell characteristics as a result of mechanical strain. There was a report that at least 1 to 2 days of cyclic strain was necessary to induce a significant increase in DNA synthesis in keratinocytes. The experiments on the changes of ECM expression during the time course is now under way. Second, we did not confirm the effect of the strained cultivated limbal epithelial cells in the in vivo environment after transplantation. Third, the effects of strain are known to be different according to the matrix to which the cells are exposed. For example, cyclic strain on collagen I and IV stimulates cell proliferation and activates focal adhesion kinases in intestinal epithelium, but plasma fibronectin inhibits these effects of strain. The matrix dependence of cyclic strain in HLECs requires further characterization in future studies.

In summary, we report the first study to investigate the effect of cyclic mechanical strain on ECM protein synthesis in HLECs. The results suggest the possibility that HLEC imposed cyclic strain during cultivation might enhance rapid migration and more stable adhesion of cells to the stroma after transplantation.

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