The role of eIF5A in epidermal growth factor-induced proliferation of corneal epithelial cell association with PI3-k/Akt activation

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Purpose: After excimer laser surgery, epidermal growth factor (EGF) plays an important role in injured corneal epithelial cell on myofibroblastic cell formation in corneal stroma. The purpose of the study is to investigate the precise mechanism of EGF on corneal wound healing, particularly on epithelial proliferation and migration.

Methods: In this study we applied small interference RNA (siRNA) to knock down the expression of eukaryotic translation initiation factor 5A (eIF5A) in corneal epithelial cells. The relative mRNA and protein expression of matrix metalloproteinase 9 (MMP9) and proliferating cell nuclear antigen (PCNA) was determined via real-time PCR and western blot analysis. The proliferative potential of EGF was evaluated via a proliferation assay using the measurement of 3H-thymidine incorporation (3H-TdR). HCEpiC apoptosis was subjected to flow cytometric analysis.

Results: The results showed eIF5A expression was enhanced and there was a statistically significant increase in EGF treatment compared to the control group. Real-time PCR, western blot analysis, and the proliferation assay demonstrated significantly lower MMP9 and PCNA expression and proliferation cell counts in eIF5A siRNA-treated groups versus significantly higher levels in EGF plus eIF5A siRNA-treated groups. The data analysis showed that eIF5A, MMP9, and PCNA expression decreased as a result of the inhibitor LY294002. Apoptotic cells were increased in the EGF plus eIF5A siRNA, EGF plus LY294002, and EGF plus eIF5A siRNA plus LY294002 groups as compared with the EGF siRNA group.

Conclusions: These results indicate that EGF-induced upregulation of eIF5A stimulates corneal epithelial cell proliferation in vitro. EGF stimulation of corneal epithelial proliferation was through the phosphatidylinositol 3-kinase (PI3-k)/protein kinase B (Akt) signaling pathway.

Photorefractive keratectomy (PRK) can result in tissue ablation with a high degree of precision and minimal damage to the adjacent tissues, and this has become a favored operation to correct refractive errors, including myopia, hyperopia, and astigmatism [1]. The operation is effective and safe, but some patients develop a postoperative wound-healing response, causing visual impairment.

The first stage of wound healing in the cornea after PRK is epithelial migration. The maintenance of the normal corneal epithelial thickness and its protective function depends on a balance between the basal layer cell proliferation at a rate adequate to replace terminally differentiated cells in the superficial layers [2]. In a variety of studies, growth factors have been identified to be involved in maintaining epithelial renewal or epithelial cell proliferation. In particular, epidermal growth factor (EGF) is a substantive contribution involved in this renewal process through its role in stimulating proliferation and differentiation [3]. An optimal dose of EGF present in the medium can repair the wound of the corneal epithelial cells at an enhanced rate. EGF stimulates wound healing in vitro by a myriad of methods. One of the events in these cascades includes the inactivation of apoptotic factors, which in turn triggers proliferation in corneal epithelial cells [4].

Eukaryotic translation initiation factor 5A (eIF5A) is highly conserved in eukaryotes and is the only known protein containing the unusual spermidine-derived amino acid residue hypusine [5]. Early observations of a correlation between hypusine synthesis and cell growth suggested an important role for hypusine in cell proliferation [5,6]. eIF5A may be a bimodular protein interacting with both RNA and proteins and is presumed to have an important role in the translation machinery [7]. Although eIF5A is intimately involved in eukaryotic cell proliferation, the true physiologic function of this essential factor has yet to be elucidated, and the potential role of eIF5A needs further investigation.

In this report we present important results to demonstrate that eIF5A upregulation is associated with EGF induction of proliferation via phosphatidylinositol 3-kinase (PI3-k)/
protein kinase B (Akt) activation. Apparently, EGF-induced human corneal epithelial cell (HCEpiC) proliferation requires upregulation of eIF5A to promote premature differentiation.

METHODS

Reagents used in experiments: The human corneal epithelial cell (HCEpiC) line was obtained from the Center of China Type Culture Collection (Wuhan, China). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). Small interference RNA (siRNA) was synthesized by Wuhan Genesil Biotechnology Co., Ltd (Wuhan, China). Human epidermal growth factor (EGF; 10 ng/ml) and LY294002 (PI3-k inhibitor) were obtained from Sigma-Aldrich Inc. (St. Louis, MO). Phototope-horseradish peroxidase (HRP) western blot detection system, including antimouse isotype laboratory of Nanjing Medical University. Unless (Gaithersburg, MD). The Cell Signaling Technology. Cell culture supplies were phospho-Akt, and anti-total Akt antibodies were products of (MMP9), proliferating cell nuclear antigen (PCNA), anti-initiation factor 5A (eIF5A), matrix metallopeptidase 9 (PI3-k inhibitor) were obtained from Sigma-Aldrich Inc. (St. Louis, MO). Phototope-horseradish peroxidase (HRP) western blot detection system, including antimouse isotype laboratory of Nanjing Medical University. Unless (Gaithersburg, MD). The Cell Signaling Technology. Cell culture supplies were purchased from Cell Signaling Technology (Beverly, MA). The annexin V- fluorescein isothiocyanate (FITC)/propidium iodide flow cytometry assay kit was purchased from Invitrogen. The antibodies for anti- eukaryotic translation initiation factor 5A (eIF5A), matrix metalloproteinase 9 (MMP9), proliferating cell nuclear antigen (PCNA), anti-phospho-Akt, and anti-total Akt antibodies were products of Cell Signaling Technology. Cell culture supplies were purchased from Gibco BRL Life Technologies Inc. (Gaithersburg, MD). The 3'H-thymidine was endowed by the isotope laboratory of Nanjing Medical University. Unless otherwise specified, all other reagents were of analytical grade.

Human corneal epithelial cell line culture and small interference RNA transfection: HCEpiCs were cultured in Dulbecco’s Modified Eagle’s Medium/Ham’s F-12 medium (Sigma-Aldrich Inc.) containing 10% fetal bovine serum and 5 μg/ml insulin in a 37 °C incubator gassed with 5% CO2. Cells used in experiments were from five to seven passages.

Lipofectin transfection of eIF5A siRNA was performed according to the vendor’s protocol: 500 pmol of eIF5A siRNA and 10 μl lipofectin were diluted in 750 μl of OptiMEM (Gibco BRL Life Technologies) in one well. After pre-incubation for 45 min at 37 °C, both solutions were mixed and incubated for an additional 15 min at room temperature. The lipofectin/eIF5A siRNA mixture was subsequently overlaid onto the HCEpiCs and incubated for 2 h. Finally, 1 ml of growth medium (20% fetal calf serum) per well was added for further cultivation of the HCEpiCs.

Construction of eIF5A small interference RNA-expressing plasmid vector: In this experiment, the targeted siRNA sequences for eIF5A were 5'-AAC GGA ATG ACT TCC AGC TGA-3'. Using pGenesil-1 as the vector backbone, the vectors of eIF5A siRNA-expressing plasmid were constructed by using green fluorescent protein (GFP) as the reporter gene. Near the 5' end of the two oligonucleotides, a BamHI and HindIII restriction site overhangs; a 6 nucleotide poly (T) tract recognized as a RNA pol III termination signal is located at the 3' end of the siRNA template. The synthesized and annealed siRNA was ligated into the BamHI and Hind 3 site of the pGenesil-1 expression vector. An unrelated gene siRNA was chosen as a negative control.

Western blot analysis: The HCEpiCs were collected with sample buffer after specific treatments. HCEpiCs were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1 mM ethylene diamine tetracetic acid (EDTA), 150 mM NaCl, 10% glycerol, 1 mM NaVO4, 50 mM NaF, 1 mM phenylmethyl sulfonylfluoride (PMSF), and protease inhibitor for 20 min on ice. After insoluble debris was precipitated by centrifugation at 13,000× g at 4 °C for 15 min, the supernatants were collected and assayed for protein concentration using the Bradford method. An equal amount of protein per sample (15 μg) was resolved on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. The transferred membranes were blocked for 1 h in 5% nonfat milk in PBS containing 135 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8 mM K2HPO4, pH 7.2, and 0.05% Tween-20 and incubated with appropriate primary antibodies and HRP-conjugated secondary antibodies. The protein bands were visualized using the enhanced chemiluminescence western detection system.

Cell proliferation assay: HCEpiCs were plated into 96-well plates and incubated overnight. The cultures were then serum starved for 24 h and treated with experimental agents for another 24 h. DNA synthesis was determined by 3'H-thymidine incorporation ('H-TdR) for the final 18 h. The media were carefully removed and the cells detached with 50 μl trypsin-EDTA. The cells were then harvested onto glass filters with a Tomtech cell harvester (LKB Wallac, San Francisco, CA), and the radioactivity retained on the dried filters was measured by the addition of 50 ml scintillation liquid and counted in a TopCount Nxt scintillation counter (LKB Wallac).

Detection of apoptotic cells: HCEpiCs apoptosis was measured on a Coulter Epics XL flow cytometer (FCM, Beckman Coulter, Erembodegen, Belgium) with apoptosis cells being annexin V positive/propidium iodide (PI) negative. HCEpiCs in a 6-well plate with a density of 1×106 were harvested and washed once with ice-cold PBS. HCEpiCs were resuspended with binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, 2.5 mM CaCl2) before being transferred to a 5-ml tube. Then cells were stained with 5 ml of annexin V-FITC for 30 min and 5 ml PI (10 μg/ml) for 15 min in the dark at room temperature. Cellular DNA was detected by FCM and apoptosis rate was computed. Annexin V and PI staining were yielded equivalent results. Data from duplicates were averaged and used as a single representation of the percentage of apoptotic cells for any given treatment.

Statistical analysis: Results are presented as means±standard deviation. Differences between various data
sets were tested for significance using the Student t test, and p values less than 0.05 were considered significant (*p<0.05; **p<0.01).

**RESULTS**

*Expression of eIF5A induced by EGF in HCEpiCs:* To investigate the relationship between the effects of EGF on the production of eIF5A in HCEpiCs, the expression of eIF5A in HCEpiCs treated with EGF (10 ng/ml) was assessed (Figure 1A,B). The results showed that the expression of eIF5A was significantly increased (p<0.01) in HCEpiCs treated with EGF when compared with the control (medium alone). This suggests that eIF5A may play an important role in the growth of EGF-treated HCEpiCs.

*Role of eIF5A on EGF-induced expression of MMP9, PCNA, and HCEpiC proliferation:* The role of eIF5A on the survival of EGF-treated HCEpiCs was investigated. Using real-time PCR and western blot analysis, the expression for MMP9 mRNA and protein was demonstrated in Figure 2A,B. Statistically significant (p<0.01) increases compared to control group were noted in the EGF group; statistically significant (p<0.05 respectively) changes compared to EGF + eIF5A siRNA were noted in eIF5A siRNA and EGF + negative siRNA. Additionally, our analysis showed that the expression cell cycle-associated protein PCNA mRNA and protein expression in EGF, EGF + eIF5A siRNA, eIF5A siRNA, EGF + negative siRNA, and medium alone group was measured in Figure 2C,D. Statistically significant (p<0.01, p<0.05 respectively) differences in mRNA and protein expression as compared to the EGF + eIF5A siRNA group were noted in the eIF5A siRNA and EGF + negative siRNA groups. The results of densitometric analyses demonstrated that HCEpiCs exposed to EGF exhibited increased MMP9 and PCNA expression when compared to the control group.

The proliferation of HCEpiCs was assessed by the changes in DNA synthesis. Figure 2E shows that EGF could significantly upregulate DNA synthesis as compared with medium alone. There was a 33% decrease in DNA synthesis in HCEpiCs exposed to EGF treated with EGF + eIF5A siRNA compared to EGF+negative siRNA and an approximately 45% decrease with eIF5A siRNA treatment compared to EGF + eIF5A siRNA for 48h after the initial treatment. This finding suggests that eIF5A may play an important role in the survival of EGF-treated HCEpiCs.

*Effect of eIF5A on EGF-induced PI3-k/Akt activation in HCEpiCs:* The effect of eIF5A on EGF-treated HCEpiCs may be involved in various signaling pathways, but which signaling pathway plays a main role is unclear. In this experiment we focused on whether the activation of the PI3-k/Akt signaling pathway in HCEpiCs needed eIF5A stimulation. Figure 3 shows that a change in phospho-Akt occurred after EGF, EGF+eIF5A siRNA, eIF5A siRNA, EGF +negative siRNA, and medium alone treatment for the defined time. The protein of phosphor-Akt was increased in the EGF group compared to the control group, and the expression of phosphor-Akt was notably changed when compared with the EGF+eIF5A siRNA and eIF5A siRNA groups. No differences
were noted between the EGF
+eIF5A siRNA and EGF
+negative siRNA groups.

The effect of LY294002 on eIF5A, MMP9, and PCNA expression and HCEpiC apoptosis: In this experiment we examined the effect of PI3-k/Akt inhibitor LY294002 on eIF5A, MMP9, PCNA, and HCEpiC apoptosis by comparing HCEpiCs treated with EGF+LY294002 to a control (EGF alone). HCEpiCs were treated with 10 µM LY294002 (PI3-k inhibitor) for 18 h, and cells were then stimulated with 10 ng/ml EGF for another 24 h. Real-time PCR results showed that treatment with LY294002 caused 51%, 78%, and 29% inhibition of eIF5A, MMP9, and PCNA mRNA expression, respectively, when compared to the control (Figure 4A). Western blot results showed that treatment caused 69%, 88%, and 65% inhibition of eIF5A, MMP9, and PCNA protein expression, respectively, when compared with the control (Figure 4B). HCEpiC apoptosis, subjected to flow cytometric analysis, showed the least number of apoptosis cells with EGF treatment when compared with other groups whereas the number of apoptotic cells by treating cells with EGF + eIF5A siRNA, EGF + eIF5A siRNA + LY294002, and EGF + LY294002 considerably increased the number of subG1 cells (Figure 4C). These results suggest that EGF-induced HCEpiC proliferation may primarily be associated with upregulation of eIF5A expression via activation of the PI3-k/Akt signaling pathway.

DISCUSSION

The renewal of the corneal epithelium is a complex process that includes the migration, proliferation, and differentiation of epithelial cells, which maintain a healthy condition of the corneal epithelium through a dynamic wound-healing process [8]. This process is largely controlled by intercellular signaling pathways through activation of a growth factor. Our
data indicate that serum-containing growth factors, such as EGF, stimulate corneal epithelial cell growth by upregulation of eIF5A expression. We investigated the relationship between the effects of EGF on the production of eIF5A. The results showed that the expression of eIF5A was significantly increased in HCEpiCs treated with EGF (Figure 1). This finding suggests that eIF5A may play an important role in the growth of EGF-treated HCEpiCs.

eIF5A is considered a nucleocytoplasmic shuttle protein, which is a multifunctional cellular protein expressed in a wide range of tissues and cell types, including lymphocytes, endothelial cells, dendritic cells, and platelets [9]. Several studies have also found a role for eIF5A involvement in cell proliferation, and more recently it has been implicated in the regulation of apoptosis [10]. Overexpression of eIF5A has been found to induce hepatocellular carcinoma proliferation [11] and skeletal stem cell differentiation [12]. To determine whether EGF correlates with the proliferative activity occurring in HCEpiC monolayers in vitro, we applied siRNA to knock down the expression of eIF5A in corneal epithelial cells. This is the first time this type of experiment has been carried out. We attempted to define the migratory mechanism of EGF-induced eIF5A expression via the MMP9 pathway. As shown in Figure 2A,B MMP9 expression increases with EGF treatment but significantly decreases with eIF5A siRNA treatment. Another aspect of re-epithelialization is the proliferation of epithelial cells behind the migrating wound front. In our study, we showed that PCNA increased expression in EGF treatment, while the expression of PCNA treated with EGF+eIF5A negative cells was greater than in treatment with EGF+eIF5A siRNA cells (Figure 2C,D). The cell proliferation assay further revealed that DNA synthesis significantly increased in EGF treatment. A statistically significant increase was noted in the EGF+negative siRNA treatment group, whereas the number of cells were lowest in the eIF5A siRNA group. Our results indicate that EGF promotes corneal epithelial proliferation and induces upregulation of eIF5A expression, which affect MMP9 and PCNA expression, and HCEpiC proliferation.

Serum-containing growth factors can induce transactivation of receptor tyrosine kinases and activate the PI3-k/Akt signaling pathway [13]. PI3-k is a heterodimeric cytoplasmic enzyme that physically associates with tyrosine-phosphorylated membrane-bound cellular proteins via the Src homology 2 (SH2) domain of itself (85 kDa regulatory subunit). Akt is one of the PI3-k effectors that play an important role in mediating transformation and anti-apoptotic effects [14,15]. These findings lead to the hypothesis that translocation and membrane localization of PI3-k are necessary for its activation in vivo. Several other studies have
also reported that PI3-k/Akt has a positive role in the EGF-induced proliferation of corneal epithelial cells [16]. Our data show that EGF can activate the PI3-k/Akt signal transduction pathway, but the expression of p*-Akt protein does not appear to change between EGF+negative siRNA and EGF+siRNA groups. Since eIF5A has no effect on the expression of p*-Akt (Figure 3), we conclude that the PI3-k/Akt signaling pathway is downstream for EGF but not for eIF5A.

Further studies are being performed to understand how the PI3-k/Akt signaling pathway is involved in EGF-induced corneal epithelial cell proliferation. Figure 4A,B shows that pretreatment with LY294002 inhibits the expression level of eIF5A, MMP9, and PCNA, suggesting that eIF5A, MMP9, and PCNA are possible substrates for the PI3-k/Akt signaling pathway in EGF-induced HCEpiC proliferation. Moreover, our data indicate that synergistic treatment with eIF5A siRNA and LY294002 significantly increases apoptotic HCEpiC death.

In summary, we have demonstrated for the first time that EGF induces HCEpiC proliferation via upregulation of eIF5A expression. The effect of eIF5A is accomplished in corneal epithelial cells through activation of the PI3-k/Akt signaling pathway, suggesting that upregulation of eIF5A is importance for EGF to elicit control of corneal epithelial cell growth and fate.
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