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Original Article

Epidermal growth factor protects against ultraviolet damage in human corneal epithelial cells through inhibiting autophagy

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Abstract: Damage of human corneal epithelial cells is often induced by exposure to various kinds of light including sunlight and artificial light. To determine the cytoprotective effects and the underlying mechanisms of epidermal growth factor (EGF) in human corneal epithelial (HCE) cells exposed to ultraviolet (UV) radiation. We found that cell viability as measured by CCK-8 assay and EDU incorporation assay was significantly decreased in HCE cells after UV exposure, while treatment with EGF promoted cell proliferation. EGF decreased the levels of LC3-I and LC3-II, the number of autophagosomes and autophagolysosomes in HCE cells exposed UV radiation. When cell autophagy was blocked by chloroquine, the cytoprotective effect of EGF was diminished as determined by cell viability assay. Furthermore, when HCE cells were treated with the ROS inhibitor NAC, cell viability was significantly decreased in EGF treated group compared with UV radiation only. In conclusion, these findings indicate that EGF has protective role in UV radiation induced HCE cell damage by inhibiting autophagy which might be mediated by increasing the production of ROS.

Keywords: Epidermal growth factor, ultraviolet radiation, autophagy, reactive oxygen species

Introduction

The eye and its corneal epithelial cells are constantly exposed to various kinds of light including sunlight and artificial light. Transmission of incident light though the eye is fundamental process for vision; however, the ultraviolet (UV) radiation poses a hazard to ocular tissues. The ultraviolet radiation is a part of the electromagnetic spectrum that covers the wavelength range from 100 to 400 nm, and is further divided into four bands: UV-vacuum from 100 to 200 nm; UV-C from 200 to 280 nm; UV-B from 280 to 315; and UV-A from 315 to 400 nm [1]. Under normal conditions, the cornea absorbs the majority of UV-B rays and protects the inner eye against UVB-induced damage [2]. Exceed absorption of UV-B might induce edema, photokeratitis, photo-ophthalmia, and epithelial damage [3]. Corneal epithelial cells are non-keratinized, stratified squamous cells that form the first defense line to protect the eye from microbial infection and various environmental hazards including the ultraviolet-induced damage [4]. The damaging cellular effects of UV radiation include DNA damage (formation of cyclobutane pyrimidinedimers), gene mutations, immunosuppression, oxidative stress and inflammatory responses, apotosis and autophagy [5]. However, therapeutic reagents for UV induced cell damage remain limited.

Many growth factors have been identified in maintaining the normal structure and function of the cornea, and in corneal epithelial healing [6, 7]. It has been reported that growth factors, such as insulin-like growth factor (IGF)-1, epidermal growth factor (EGF), interleukin 6, fibroblast growth factor-2, transforming growth factor-b (TGF-b), keratinocyte growth factor (KGF) and hepatocyte growth factor could stimulate corneal epithelial cell migration both in vivo and

in vitro [8]. Of note, EGF, as a physiological ingredient in the tears, was reported to increase the proliferation and differentiation of corneal keratocytes and endothelial cells, contributing to the regeneration of corneal epithelium [9]. Evidence for the protective effect of EGF in UV induced cell injury remains limited and the underlying mechanisms need to be investigated.

Macroautophagy (herein termed autophagy) is a cellular stimulatable self-catabolic process that constitutively clears damaged proteins and organelles to an autolysosomal compartment for degradation via lysosome-dependent pathway, which is critical in maintaining homeostasis under physiological conditions [10]. Autophagy is observed in process of development, differentiation, and tissue remodeling in various organisms [11]. However, whether autophagy plays beneficialor detrimental role remains controversial. Autophagy dysfunction is correlated with diverse pathologies, such as neurodegeneration, cancer, infection and aging, eye diseases, and vascular disorders [12]. Enhancing cell autophagy or reducing that process has become therapeutic target depending on the situations.

Herein, the present study was designed to investigate whether EGF protects against UV-induced cell damage in human corneal epithelial cells. Our results demonstrate that EGF can significantly improve cell viability, which could possibly mediated by reduction of autophagy, and reactive oxygen species (ROS) contribute to the cytoprotective effect of EGF.

Materials and methods

Reagents

All cell culture reagents were purchased from Invitrogen (Gibco BRL, Burlington, ON, Canada). A cell counting kit-8 (CCK-8) was acquired from Dojin Laboratories (Kumamoto, Kyushu, Japan). DAPI was obtained from Invitrogen/Life Technologies (Carlsbad, CA, USA).

Cell culture and UV irradiation

Human corneal epithelial (HCE) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultivated on 6-well cell culture plates in Keratinocyte-SFM medium (Gibco, Invitrogen, Paisley, UK) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco), and 10% fetal bovine serum (HyClone, Logan, UT) in a humidified incubator constantly filled with 5% CO_2 at 37°C. For UV irradiation experiments, cells reached approximately 85%-90% confluence, the growth medium was removed and the cells were at a distance of 60 cm from the UV light source and exposed at an intensity of 42 μJ/cm^2.

Cell viability assay

The viability of the HCE cells was evaluated by CCK-8 assay. 5×10^3 cells were seeded into 96-well plates. Briefly, after the treatment of the different groups, 10 μL of CCK-8 reagent was added to the cells, followed by incubation for 1.5 h at 37°C with 5% CO_2. The optical density (OD) was measured at an absorbance wavelength of 450 nm with a multifunctional microplate reader (Thermo) according to the manufacturer's instructions.

Western blot

HCE cells were washed with pre-cold PBS and lysed with the RIPA buffer (50 mmol/L HEPES at pH 7.5, 150 mmol/l NaCl, 10% glycerol, 1.5 mmol/L $MgCl_2$, 1% Triton-X 100, 1 mmol/L EDTA at pH 8.0, 10 mmol/L sodium pyrophosphate, 10 mmol/L sodium fluoride) supplemented with the protease and phosphatase inhibitor cocktails, and centrifuged at 1,2000 g for 10 min at 4°C. After quantification of protein concentrations by BCA protein assay kit, equal amount of protein (30 μg) was subjected to SDS-PAGE, and followed by transferred onto PVDF membrane at 400 mA for 1-2 h at 4°C. Membranes were blocked with 5% non-fat milk and probed with indicated primary antibody overnight at 4°C and then blotted with indicated secondary antibodies. Membranes were developed with enhanced-chemiluminescence substrate and visualizedwith Bio-Rad Chemi-DocTM XRS system (Hercules, CA, USA). Intensities of proteins were analyzed using Image Lab 3.0 (BioRad, Hercules, CA, USA). Monoclonal antibody against β-actin was used as the internal control.

Transmission electron microscopy (TEM)

Cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer. The cells were washed with 0.1 M sodium cacodylate buffer

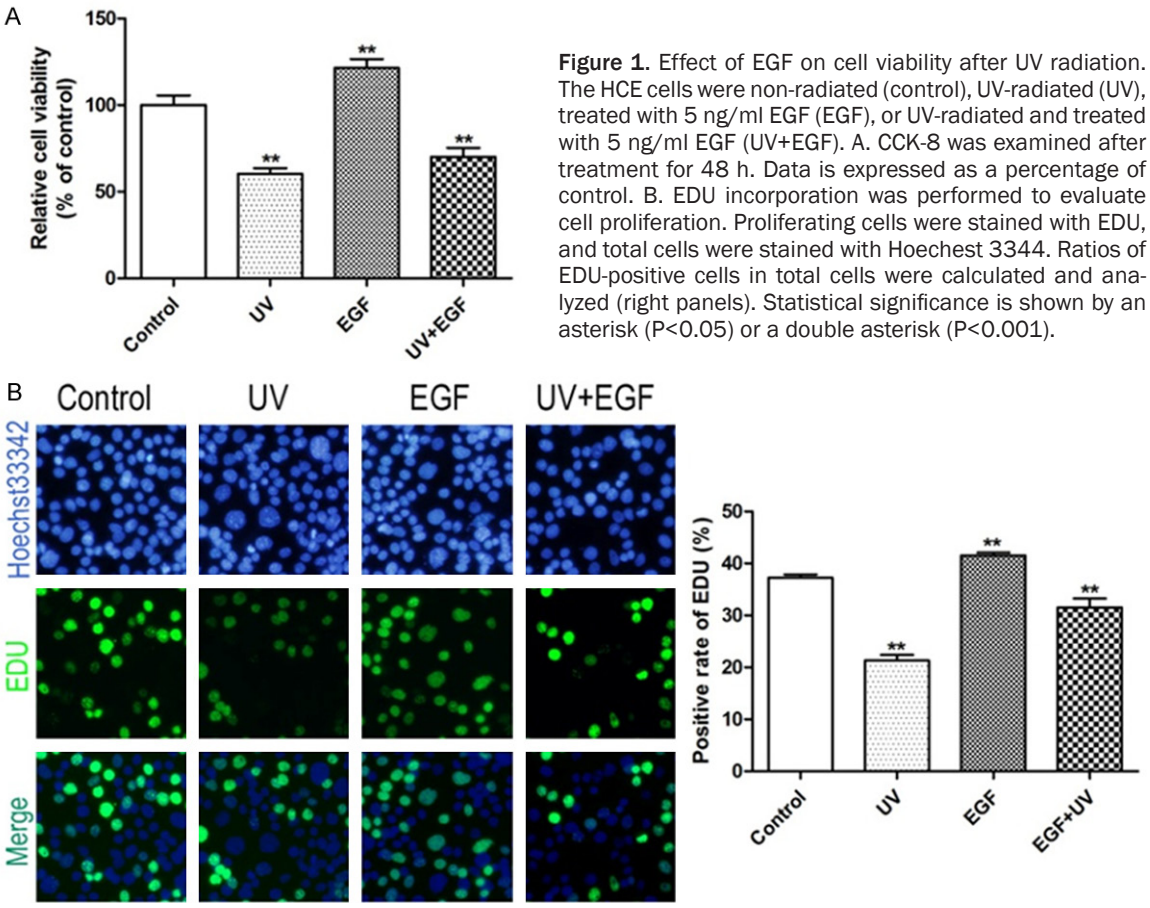

Figure 1. Effect of EGF on cell viability after UV radiation. The HCE cells were non-radiated (control), UV-radiated (UV), treated with 5 ng/ml EGF (EGF), or UV-radiated and treated with 5 ng/ml EGF (UV+EGF). A. CCK-8 was examined after treatment for 48 h. Data is expressed as a percentage of control. B. EDU incorporation was performed to evaluate cell proliferation. Proliferating cells were stained with EDU, and total cells were stained with Hoechest 3344. Ratios of EDU-positive cells in total cells were calculated and analyzed (right panels). Statistical significance is shown by an asterisk ($P<0.05$) or a double asterisk ($P<0.001$).

and postfixed with 1% osmium tetroxide. The specimens were then dehydrated in graded ethanol series, infiltrated, and embedded in Spurr's resin. TEM was performed on PHILIPS CM120 TEM at an accelerating voltage of 120 Kv. Images were acquired with Gatan type UltraScan 4000SP CCD Camera connected to the TEM.

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Comparisons between two groups were evaluated by Student's *t*-test. Values of $P<0.05$ were considered statistically significant. Assays were performed at least three times independently.

Results

EGF enhances HCE cell proliferation in response to UV radiation

To examine the effect of EGF in UV-induced cell damage, HCE cells were divided into the following four groups: control, UV, EGF, and UV+EGF group. As shown by CCK-8 assay, in comparison to the control group, UV-treatment significantly inhibited cell proliferation while EGF treatment showed opposite effect (**Figure 1A**). EGF could enhance the UV-induced cell proliferation inhibition (**Figure 1A**). EDU-incorporation results confirmed the effect of EGF, where UV+EGF treated cells displayed elevated percentage of EDU-incorporation compared to UV treated cells (**Figure 1B**).

EGF resuces UV radiation induced HCE cells autophagy

To explore the potential mechanism of EGF mediated cell proliferation, autophagy was examined. LC3 is a well-established marker of autophagosomes in mammalian cells. In this study, HCE cells were tansfeced with mRFP-GFP-LC3 and subjected to the UV-radiation or EGF treatment 24 hours later. HCE cells in UV group showed significantly increased autophagy compared to the other three groups; and EGF significantly reduced the rate of autophagy in response to UV radiation (**Figure 2A**). The for-

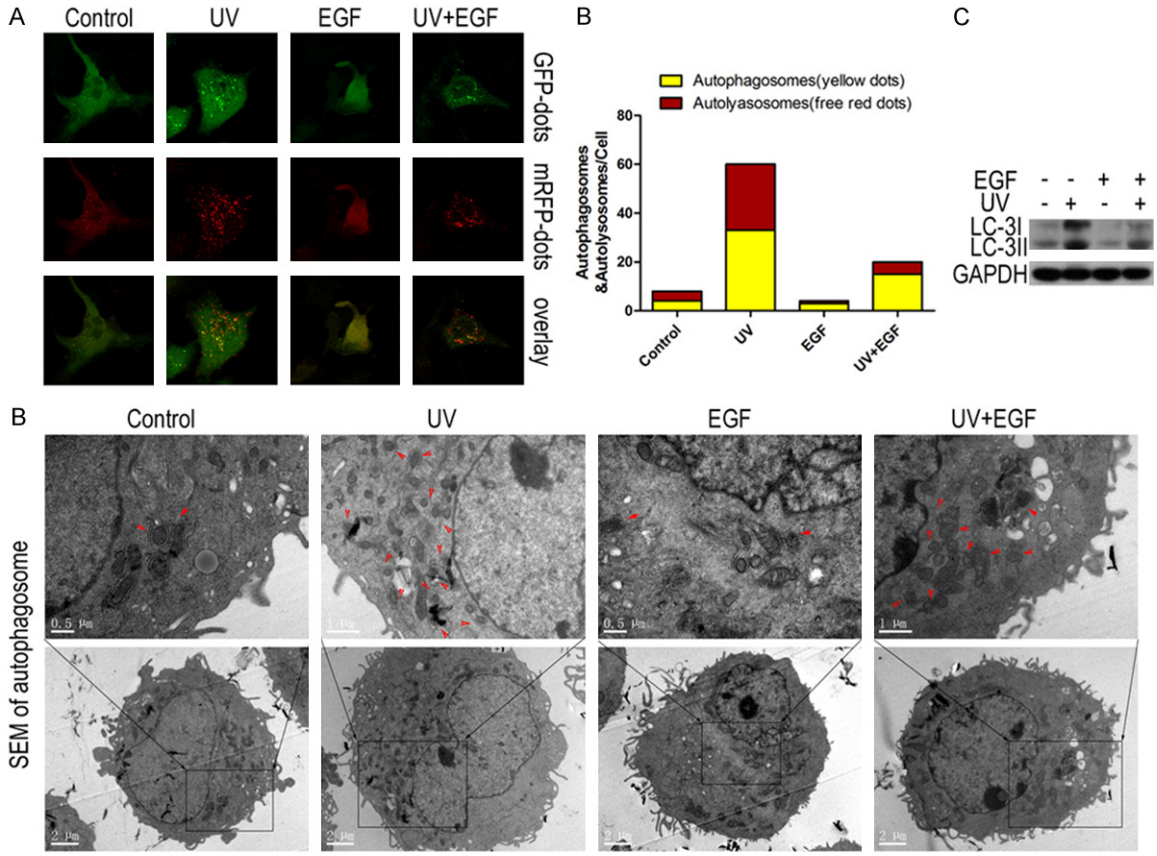

Figure 2. Effect of EGF on cell autophagy after UV radiation. The HCE cells were non-radiated (control), UV-radiated (UV), treated with 5 ng/ml EGF (EGF), or UV-radiated and treated with 5 ng/ml EGF (UV+EGF). HCE cells were treated with EGF for 24 h. A. HCE cells were transfected with mRFP-GFP-LC3 plasmid. 24 h after transfection, HCE cells were subjected into different treatment. Representative fluorescence images were shown (left panels). Autophagosomes and autolysosomes were quantified and analyzed (right panels). B. Autophagic vacuoles were examined by transmission electron microscopy. Arrows indicate autolysosomes (double membrane structure). C. Western blot was performed to examine the levels of LC3-I and LC3-II with GAPDH as loading control.

mations of autophagosomes and autophagolysosomes are important signs of autophagy. So, we observed the formations of autophagosomes and autophagolysosomes by transmission electron microscopy (TEM). Autophagosomes are double-membrane autophagic vacuoles which contain oligomeric protein complexes and organelles. Autophagolysosomes are single-membrane bound degradative vacuoles that are formed by fusion of autophagosomes with lysosomes [11, 13]. In this study, HCE cells in exposure to UV radiation showed significantly increased levels of autophagosomes and autophagolysosomes (**Figure 2B**). By contrast, cells treated with EGF showed reduced autophagy, as revealed by autophgosomes and autophagolysosomes (**Figure 2B**). Notably, when HCE cells received EGF treatment after UV radiation, they displayed significantly decreased levels of autophgosomes and autophagolysosomes (**Figure 2B**). When autophagy occurs, punctate LC3 protein appears, and the soluble form of LC3 (LC3-I) is converted into the lipidated and autophagosome-associated form (LC3-II) [14]. In the present study, we showed EGF treated cells which were exposed to UV radiation had decreased LC3 autophagy flux. UV treated cells had increased levels of LC3-I (cytosolic) and lipidated LC3-II, whereas EGF treated cells showed evidently decreased levels of both LC3-I and LC3-II (**Figure 2C**). Together, these data suggested that EGF could significantly attenuate the autophagy flux of cells which might be trigged by UV radiation.

Inhibition of autophagy by chloroquine abrogates cell proliferation inhibitory effect of UV condition

To determine whether autophagy displayed protective roles in the UV radiation induced cell

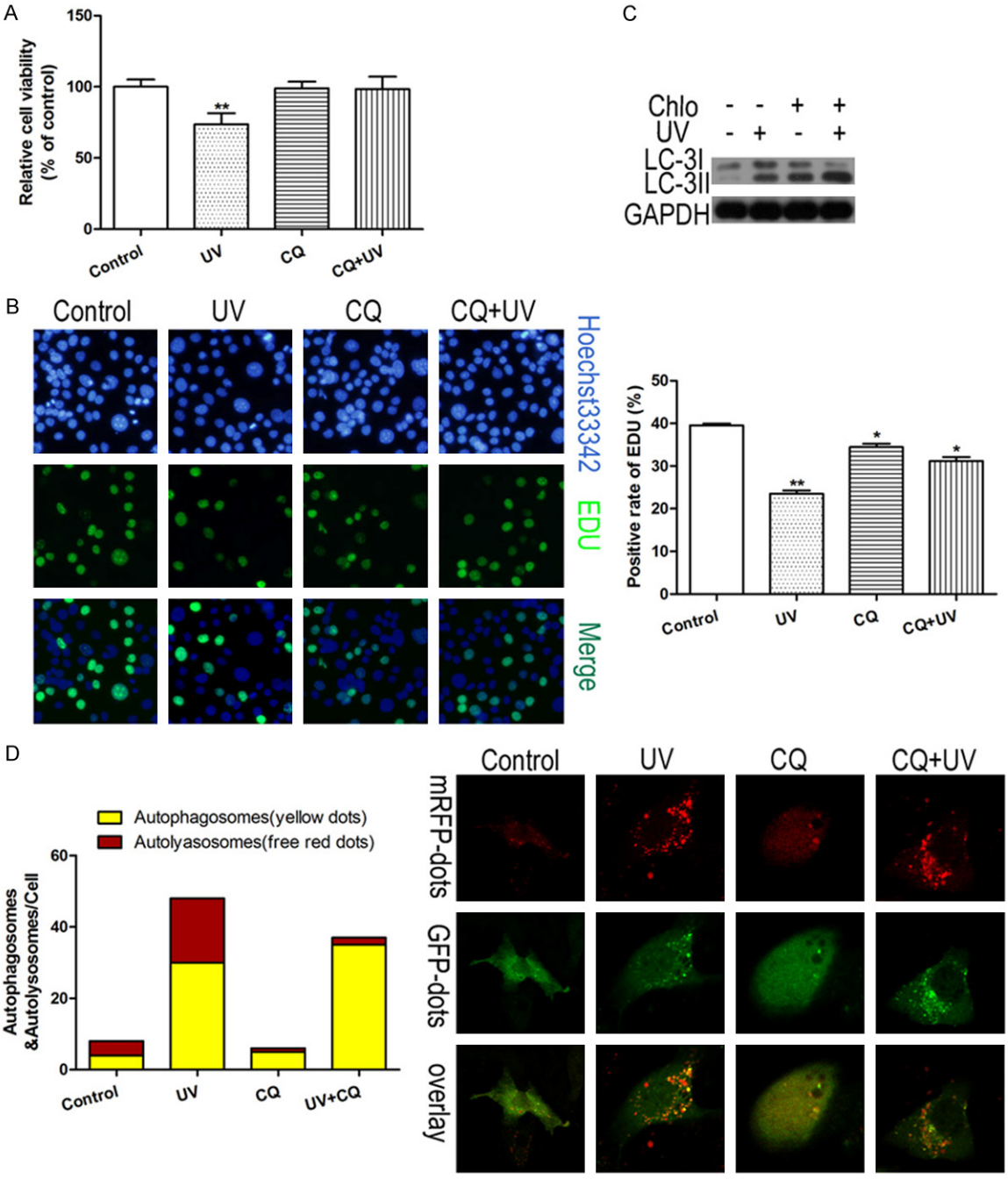

Figure 3. Effect of inhibiting autophagy by chloroquine on cell viability and autophagy after UV radiation. The HCE cells were non-radiated (control), UV-radiated (UV), treated with 10 μmol/L chloroquine (CQ), or UV-radiated and treated with 10 μmol/L CQ (UV+CQ). A. CCK-8 was examined after treatment for 48 h. Data is expressed as a percentage of control. B. Cell proliferation was detected by EDU incorporation assay after treatment for 24 h. Ratios of EDU-positive cells in total cells were calculated and analyzed (right panels). C. Western blot analysis of cell lysates was performed using LC3-I and LC3-II antibodies. GAPDH was used as internal control. D. After HCE cells were transfected with mRFP-GFP-LC3 plasmid for 24 h, cells were subjected into different treatment. Representative fluorescence images were shown (right panels). Autophagosomes and autolysosomes were quantified and analyzed (left panels). Statistical significance is shown by an asterisk ($P<0.05$) or a double asterisk ($P<0.001$).

proliferation inhibition, a well-known autophagy inhibitor, chloroquine (CQ), was employed in this study. Cells were divided into control, UV, CQ, UV+CQ groups. The CCK-8 results showed

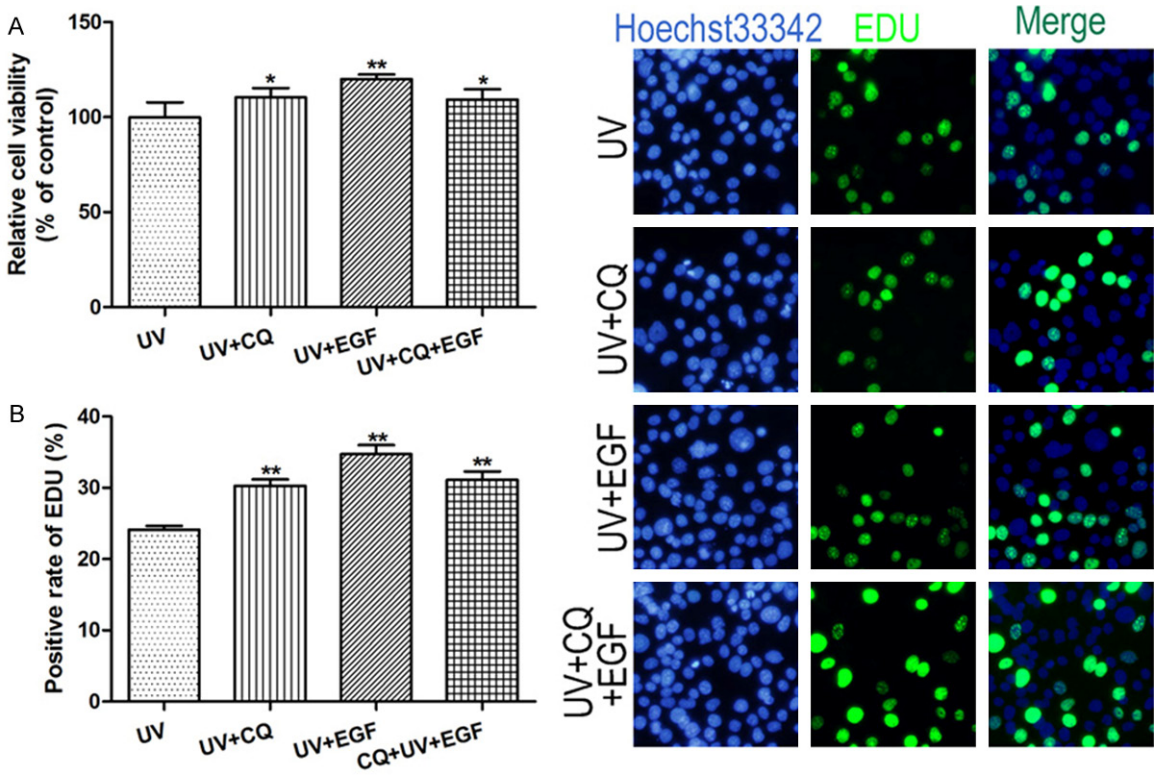

Figure 4. Effect of inhibition of autophagy by chloroquine on the role of EGF after UV radiation. The HCE cells were UV-radiated (UV), UV-radiated and treated with 10 μmol/L chloroquine (UV+CQ), UV-radiated and treated with 5 ng/ml EGF (UV+EGF), or UV-radiated and treated with 10 μmol/L chloroquine and 5 ng/ml EGF (UV+CQ+EGF). A. CCK-8 was examined after treatment for 48 h. Data is expressed as a percentage of control. B. EDU incorporation was performed to evaluate cell proliferation. Proliferating cells were stained with EDU, and total cells were stained with Hoechest 3344. Ratios of EDU-positive cells in total cells were calculated and analyzed (right panels). Statistical significance is shown by an asterisk ($P<0.05$) or a double asterisk ($P<0.001$).

that the cell growth of HCE cells was higher after chloroquine administration for 48 h after UV exposure for 30 s compared to UV exposure only (**Figure 3A**). The effect of chloroquine was further confirmed by EDU incorporation experiment. Similarly, HCE cells in CQ+UV group showed more EDU-positive rates than cells in UV group (**Figure 3B**). Furthermore, we examined the autophagy inhibition role of chloroquine by Western Blot andtwo-color fluorescence assay. Western Blot results revealed that cells in CQ+UV group showed decreased level of LC3-I and increased level of LC3-II compared with cells in UV group (**Figure 3C**). In addition, chloroquine treatment resulted in decreased levels of autophagosomes and autolysosomes in HCE cells exposed to UV when compared with cells exposed to UV (**Figure 3D**). Together, these results suggested that inhibition of autophagy by chloroquine could attenuate the autophagic damage caused by UV exposure.

Blockade of autophgy by chloroquine attenuates the protective effect of EGF in the process of UV radiation induced damage

To verify our hypothesis that the protective effect of EGF was partly mediated by autophagy, we used EGF in combination with chloroquine. In this part, cells were allocated into UV, UV+CQ, UV+EGF, UV+CQ+EGF. Cell proliferation was determined after HCE cells were exposed to UV radiation and then treated with indicated drugs for 48 h. CCK-8 assay showed that no significant difference of HCE cell proliferation was observed between UV+CQ+EGF and UV+CQ groups, suggesting that the protective effect of EGF was abolished by autophagy inhibitor, chloroquine (**Figure 4A**). Similar results were obtained by EDU incorporation assay, where HCE cells in UV+CQ+EGF and UV+CQ groups showed almost the same EDU-positive rates (**Figure 4B**). Collectively, blockage of autophagy by pharmacological inhibitor

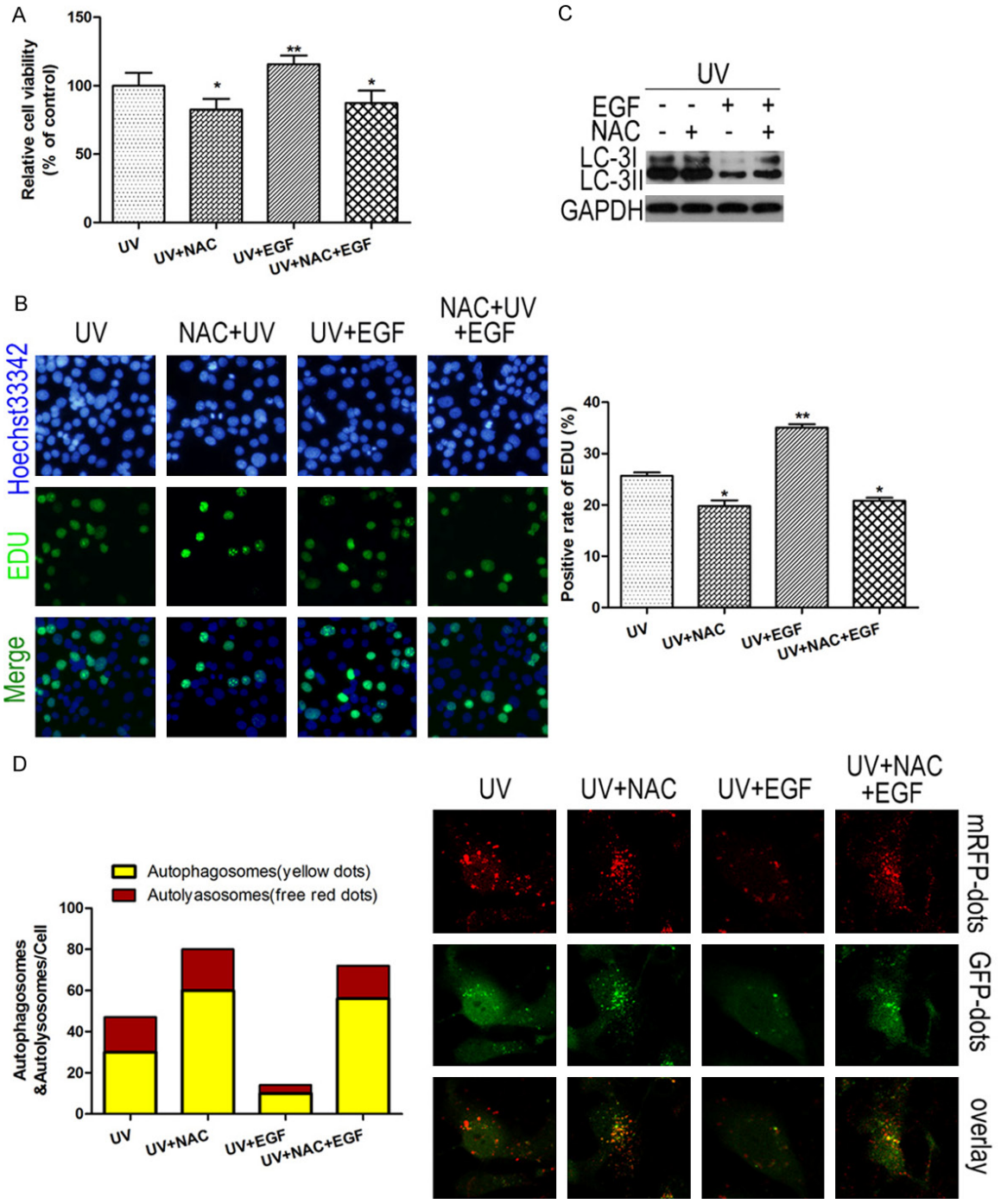

Figure 5. Effect of inhibition of ROS by N-acetylcysteine on the role of EGF after UV radiation. The HCE cells were UV-radiated (UV), UV-radiated and treated with 40 mmol/L NAC (UV+NAC), UV-radiated and treated with 5 ng/ml EGF (UV+EGF), or UV-radiated and treated with 40 mmol/L NAC and 5 ng/ml EGF (UV+NAC+EGF). A. Cell viability was determined with CCK-8 assay in different conditions. CCK-8 was performed 48 h after treatment with NAC for 30 min. B. EDU incorporation was performed after HCE cells were treated with NAC for 24 h. Ratios of EDU-positive cells in total cells were calculated and analyzed (right panels). C. LC3-I and LC3-II levels in different conditions were analyzed with Western blot. D. 24 h after HCE cells were transfected with mRFP-GFP-LC3 plasmid, cells were subjected into different treatment. Representative fluorescence images were shown (right panels). Autophagosomes and autolysosomes were quantified and analyzed (left panels). Statistical significance is shown by an asterisk ($P<0.05$) or a double asterisk ($P<0.001$).

suppressed protective effect of EGF in UV induced cell damage, revealing that autophagy was required for UV radiation induced cell damage and could be attenuated by EGF.

ROS is involved in the EGF mediated autophagy reduction in UV radiation induced cell damage

Our previous study showed that EGF could promote the production of reactive oxygen species (ROS). However, whether ROS was involved in the process of autophagy inhibition by EGF in the UV radiation induced cell damage was unknown. Herein, we investigated the role of ROS in the process by using N-acetylcysteine (NAC), a typical ROS scavenger. Cells were divided into four groups: UV, UV+NAC, UV+EGF, UV+NAC+EGF. Protective effect of EGF was diminished when ROS production was inhibited by NAC, as revealed by decreased cell viability in UV+NAC+EGF group compared with UV+EGF group by CCK-8 assay (**Figure 5A**). Cell proliferation was further examined by EDU incorporation assay, which showed that HCE cells in UV+NAC+EGF group showed relatively lower EDU positive rate in comparison with those in UV+EGF group (**Figure 5B**). However, whether NAC caused inhibition of ROS was correlated with autophagy remained unclear. We detected the levels of LC3-I and LC3-II by Western Blot. Our results showed that HCE cells in UV+NAC+EGF group had higher levels of LC3-I and LC3-II compared to UV+EGF group, suggesting that inhibition of ROS could enhance cell autophagy (**Figure 5C**). Fluoresence results also indicated increased autophagy was found in UV+NAC+EGF group when compared with UV+EGF group (**Figure 5D**). Taken together, these data indicated that the protective effect of EGF was mediated, at least for a part, by production of ROS.

Discussion

Autophagy plays fundamentally important role for the maintenance of intracellular homeostasis and is essential for the degradation of intracellular components via a lysosome-dependent pathway [15, 16]. Although autophagy serves as a cytoprotective mechanism in response to stress, it can also lead to cell death under specific circumstances, a process known as 'autophagic cell death' (ACD), which is distinguished from the other form of programmed cell death [17]. ACD is morphologically defined as a type of cell death that occurs in the absence of chromatin condensation but is accompanied by large-scale autophagic vacuolization of the cytoplasm [18]. Thus, the balance between the production of autophagosome and appropriate lysosomal degradation determines the protective or detrimental role of autophagy.

In the present study, we demonstrated that autophagy was impaired in HCE cells during the process of UV radiation induced damage and that inhibition of autophagy by chloroquine attenuated the protective effect of EGF at UV circumstance. Furthermore, inhibition of the production of ROS abolished the protective role of EGF, indicating that the effect is mainly due to increased production of ROS.

To address the role of EGF in the UV induced damage, we measured cell viability via CCK-8 and EDU incorporation assays. As with previously studies, we found UV radiation could result in a significantly reduction in HCE cell viability. When HCE cells were treated with EGF, they showed significantly an increase of cell viability compared with control cells. And EGF treatment could increase cell viability after UV exposure. These data suggested that EGF played protective effect during UV radiation induced cell damage.

In order to study the effect of EGF on the autophagy process, we tansfected HCE cells with mRFP-GFP LC3 plasmid, and analyzed the levels of autophagosomes and autolysosomes. After treatment with EGF, the amount of autolysosmes showed a remarkably decreased, indicating a defect of autophagy by EGF treatment. Accordingly, we analyzed the protein level of LC3, a key autophagy marker. We observed that the level of LC3-I and LC3-II in HCE cells treated with EGF showed an obviously decrease compared to those treated with UV radiation. When HCE cells received UV radiation, followed by treatment with EGF, they showed decreased levels of both LC3-I and LC3-II, suggesting EGF could decrease the level of autophagy in UV induced damage. Moreover, our TEM results were in consistent with Immunomicroscopy and Western Blot, confirming the role of EGF in autophagy reduction process.

To confirm that EGF displayed protective role in the UV radiation induced cell damage by inhibition of autophagy, we usedchloroquine as a model to investigate autophagy process in this study. The CCK-8 and EDU incorporation experiment results showed that HCE cells prolifera-

tion was higher after chloroquine administration under UV radiation condition. And autophagy marker, autophagosomes and autolysosomes were downregulated by choloroquine treatment in HCE cells after UV exposure. In addition, the protective effect of EGF, as measured by CCK-8 and EDU incorporation assay, was abolished by chloroquine. Collectively, blockage of autophagy by pharmacological inhibitor attenuated the protective effect of EGF in UV induced cell damage, revealing that autophagy was required for UV radiation induced cell damage and could be attenuated by EGF.

Endogenous reactive oxygen species (ROS) include hydroxyl radical, superoxide anion, and hydrogen peroxide, and are mainly produced on the mitochondrial inner membrane during the process of oxidative phosphorylation via the electron transport chain [19, 20]. Oxidative stress plays vital roles in the pathogenesis of many diseases, including ocular surface diseases, such as keratoconus, Fuchs' dystrophy [21-26]. It is well established that continuous exposure to UV irradiation causes excessive production of ROS [27-29]. Extremely high levels of ROS destroy almost all cellular components, which then trigger cell death [30]. However, mild level of ROS might play opposite roles.

In the present study, we investigated whether EGF reduced UV radiation induced autophagy by promoting generation of ROS. Given the evidence that ROS generation can be suppressed by the classic antioxidant, N-acetylcysteine, we used NAC as ROS scavenger. Cell viability showed no significant changes when HCE cells were treated with a combination of NAC and EGF compared to those treated with EGF only under UV radiation condition, as measured by CCK-8 or EDU incorporation experiments. Evidence has already demonstrated that an increase in ROS level in mitochondrion is correlated with autophagy defect [31]. Hence, we detected whether the anti-autophgic role of EGF was mediated by ROS. The levels of LC3-I and LC3-II by Western Blot, which showed that HCE cells in UV+NAC+EGF group had higher levels of LC3-I and LC3-II compared to UV+EGF group, suggesting that inhibition of ROS could enhance cell autophagy. Furthermore, Immnunomicroscopy results also indicated increased autophagy was found in UV+NAC+EGF group when compared with UV+EGF group. Thus these data firmly indicated that EGF showed anti-autophygic role in UV induced damge by increasing the generation of ROS.

In conclusion, this study demonstrated that EGF promotes HCE cells survival by autophagy inhibition, and these biological changes can be reverted by cholorquine. What's more, the autophagy inhibitory effect of EGF is mediated by generation of ROS. Taken together, our data support EGF has therapeutic potential in UV radiation related corneal diseases.

Acknowledgements

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Disclosure of conflict of interest

None.

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