Original Article

The protective effect of simvastatin against ultraviolet B-induced corneal endothelial cell death

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Purpose: Excessive ultraviolet B (UVB) exposure causing corneal endothelium injury, including apoptosis, is a serious condition. Therefore, drugs that can inhibit apoptosis in corneal endothelial cells represent an effective strategy. Simvastatin is widely used as a specific inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase, can reduce levels of low density lipoprotein (LDL) cholesterol, and exerts anti-inflammatory effects. However, the protective effect of simvastatin on corneal endothelial cells remains unclear. Therefore, the aim of this study was to elucidate whether UVB promotes the initiation of apoptosis in corneal endothelial cells and injury reversible by simvastatin treatment. **Methods:** We detected the cell viability, subG1 population, and caspase-3 activity. **Results:** Results showed that simvastatin alleviates UVB-induced cell death, cell apoptosis, and caspase-3 activity. **Conclusion:** Our findings indicated that simvastatin alleviated UVB-induced corneal endothelial cell apoptosis via caspase-3 activity.

Key words: Apoptosis, caspase-3 activity, corneal endothelium cells, simvastatin, ultraviolet B

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Corneal edema is a common characteristic of acute or protracted corneal disease of various etiologies. Corneal transparency is primarily dependent on the ability of the cornea to remain in a dehydrated state. Importantly, the function of corneal endothelial cells is a major determinant of corneal hydration.[1] Clinically, the corneal endothelial cell density has been considered a key indicator for evaluating and maintaining corneal health.[2-4]

Ultraviolet (UV) light is the most common cause of radiation injury to the eye. The major function of the cornea is to absorb the majority of UV radiation. UV radiation damage to the corneal epithelium is cumulative, and UVB radiation presents a higher risk of pathogenesis than UVA.[5-7] Increasing evidence indicates that UVB radiation from sunlight induces corneal inflammation or photokeratitis accompanied by changes in corneal hydration.[8,9] Moreover, it has also been reported that exposure to high doses of UVB can cause corneal endothelium damage and may cause fluctuating or occasionally blurred vision.[8,10,11] Therefore, preventing corneal endothelium degradation is considered an effective strategy for treating corneal edema.

Statins are potent cholesterol-lowering agents with broad anti-inflammatory properties.[12] Morimoto *et al*. reported that lovastatin enhances the clearance of apoptotic cells in chronic obstructive pulmonary disease.[13] Moreover, several studies have demonstrated that simvastatin exerts anti-inflammatory and antifibrotic effects in acute lung injury, inflammatory arthritis, and bleomycin-induced pulmonary fibrosis.[13-16] However, the effect of simvastatin in eye diseases remains unclear. Therefore, in this study, we examined the effect of simvastatin on corneal endothelial cells following UVB irradiation.

Methods

Cell culture

Bovine corneal endothelial (BCE) C/D-1b cells were procured from Bioresource Collection and Research Center and cultured under optimized conditions according to the supplier's instructions. Briefly, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, New York, NY, USA) supplemented with 10% FBS, 25 mM D-glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin-streptomycin (50 U/mL; Sigma, St. Louis, MO, USA) at 37°C in 5% CO_2/95% air. The culture medium was replaced with fresh medium on alternate days. Once the cells reached 70%–80% confluence, they were trypsinized and seeded onto 6- or 24-well plastic culture dishes for subsequent experiments.

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Cell viability assay

Cell viability was measured using a WST-1 assay. Cells were seeded at a density of 5 × 10^4 cells/ml in 24-well plates and cultured in phenol red-free DMEM containing 0.5% heat-inactivated FBS for 24 h. Next, cells were incubated with indicated concentrations of simvastatin for 24 h. The WST-1 reagent was then added into the medium and the cells were incubated at 37°C for 2 h. The absorbance was measured at 450 nm in a microplate reader (BIO-RAD, Hercules, CA, USA).

Cell cycle analysis

Cells were treated with or without simvastatin in the presence or absence of UVB (10 mJ/cm^2) for 24 h before being harvested and fixed in ice-cold 70% ethanol for 1 h. Cells were then washed with phosphate-buffered saline and incubated in propidium iodide staining buffer (50 μg/ml propidium iodide, 0.1 mg/ml DNase-free RNase A, and 0.5% Triton X-100) for 30 min at 37°C in the dark. The DNA content was analyzed by flow cytometry (BD Biosciences, CA, USA).

Caspase-3 activity assay

The activity of caspase-3 was assayed using a caspase-3/CPP32 colorimetric assay kit. Briefly, cells were treated with oridonin for 2 h and then resuspended in cell lysis buffer (BioVision, Milpitas, CA, USA). Samples were centrifuged at 10,000 g for 5 min at 4°C to yield cell lysates. Protein concentrations of cell lysates were determined by the Bradford method using bovine serum albumin as a standard. The isolated supernatant was mixed with an equal volume of reaction buffer (containing 10 mM DTT and 400 μM DEVD-pNA) and incubated at 37°C for 120 min. The absorbance was measured at 450 nm in a microplate reader (BIO-RAD, USA).

Statistical analysis

Data are expressed as the mean ± standard error of the mean, and comparisons were statistically calculated by one-way or two-way analysis of variance followed by a Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Simvastatin alleviates ultraviolet B-induced cell death

UVB significantly induced cell death in BCE C/D-1b corneal endothelial cells according to the cell viability analysis [Fig. 1]. To determine the effect of simvastatin on cell survival, cell viability was determined in cells treated with 0.5 μM simvastatin before UVB irradiation. Results showed that simvastatin significantly inhibited UVB-induced cell viability [Fig. 1].

Simvastatin inhibits ultraviolet B-induced endothelial cell apoptosis and caspase-3 activity

To further determine whether UVB-induced cell death through apoptosis, cell cycle, and caspase-3 activity were evaluated. UVB caused an increase in cells in the sub-G1 phase, which is an indicator of apoptosis. Furthermore, simvastatin reversed the UVB-induced increase in the sub-G1 population [Fig. 2].

Caspase-3 plays a central role in the apoptotic response. Our results showed that caspase-3 activity was significantly increased after UVB irradiation, but that this effect was reversed in the presence of simvastatin [Fig. 3]. These findings indicate that UVB-induced apoptosis in BCE C/D-1b corneal endothelial cells through a caspase-3 dependent pathway.

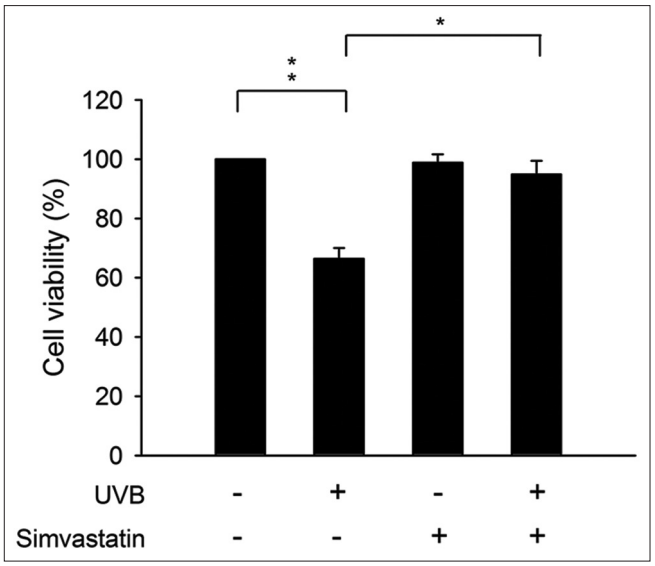

Figure 1: Simvastatin reversed ultraviolet B-induced cell death. Cells were treated with control or 0.5 μM simvastatin for 24 h before ultraviolet B exposure. Following exposure for 24 h, cell viability was determined using a WST-1 assay. All data are presented as the mean ± standard error of the mean ($n = 3$). *$P < 0.05$, **$P < 0.01$

Discussion

The importance of taking appropriate precautions to protect the eyes from the increasing levels of UV radiation reaching the Earth's surface through stratospheric ozone layer depletion has been established.[17] Recent studies demonstrated that corneal epithelial stem cells reside preferentially in the basal layer of the peripheral cornea in the limbal zone, rather than uniformly in the entire corneal epithelium.[18,19] Therefore, it is imperative to develop agents capable of preventing corneal damage caused by UVB irradiation.

Simvastatin is a widely used specific inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase and can reduce the level of low-density lipoprotein cholesterol as well as exert anti-inflammatory effects.[20,21] A previous clinical study has demonstrated that simvastatin may be associated with visual field stabilization in patients with normal tension glaucoma. A larger scale randomized controlled trial and cost-effective analyses seem to be approved.[22] Moreover, statins have been shown to reduce ocular inflammation in animal models of uveitis and to prevent the development of uveitis in observational studies.[23] Shirinsky *et al*. reported that simvastatin may have therapeutic potential in noninfectious uveitis; however, these results need to be confirmed in double-blinded, randomized, and controlled studies.[24] Furthermore, Harris *et al*. reported a lack of strong evidence of any adverse effects on lens opacity formation following 18 months of simvastatin treatment.[25] Therefore, the effect of simvastatin in eye diseases remains conflicting.

UVB overexposure is the most common cause of radiation damage to the eyeball and is a risk factor for human corneal damage.[26,27] Photokeratitis and UV keratitis are eye diseases associated with UVB-induced corneal damage. The cellular and molecular mechanisms involved in photokeratitis and UV keratitis have already been reported.[28-30] Accumulating evidence reported that UVB-induced corneal cell apoptosis *in vitro* and

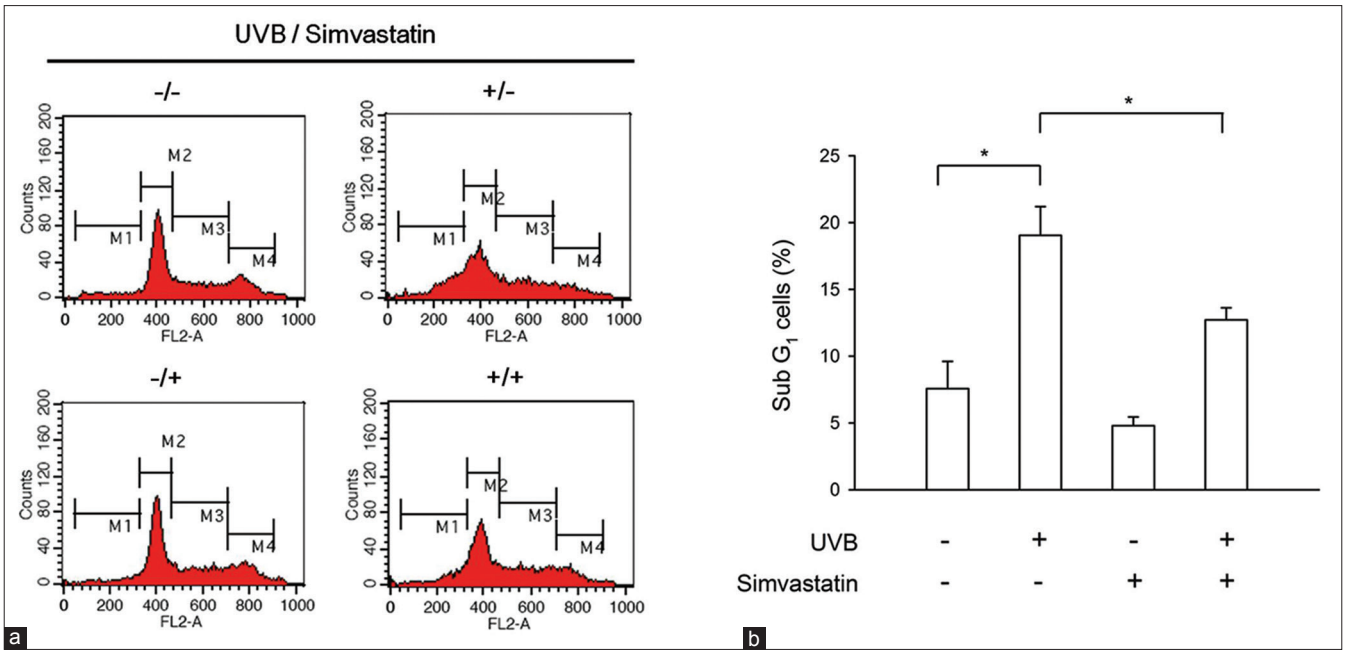

Figure 2: Protective effect of simvastatin against ultraviolet B-induced cell apoptosis. (a) Changes in the sub-G1 phase population were determined following treatment with control or simvastatin (0.5 μM) before ultraviolet B exposure. (b) Representative sub-G1 populations calculated from flow-activated cell sorting histograms are shown ($n = 3$). All data are presented as the mean ± standard error of the mean ($n = 3$). *$P < 0.05$

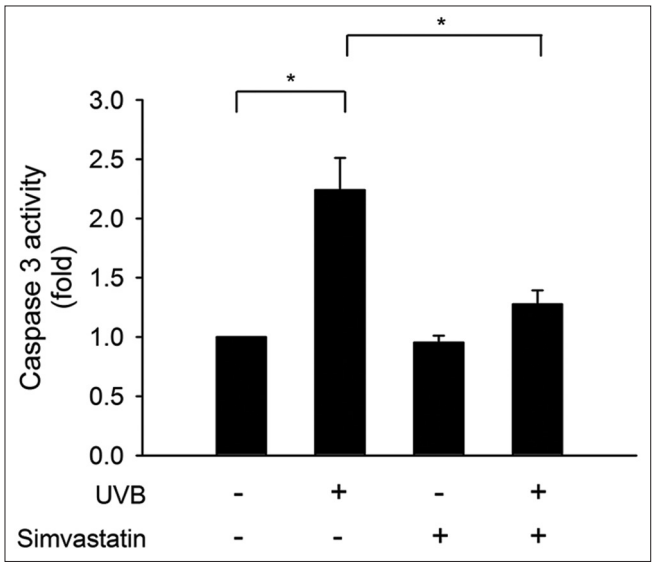

Figure 3: Simvastatin inhibited ultraviolet B-induced caspase-3 activity. Cells were treated with control or 0.5 μM simvastatin for 24 h before ultraviolet B exposure for a further 24 h. Caspase-3 activity was measured using a caspase-3/CPP32 colorimetric assay kit ($n = 3$). *$P < 0.05$

in vivo.[31-34] Moreover, many studies[35-41] have revealed that UVB radiation causes mouse corneal tissue abnormalities. Therefore, studying of the potential protective molecule is important in developing better remedial strategies for corneal endothelial cell damage through apoptosis. The aim of this study was to investigate the protective effects of simvastatin against UVB-induced corneal apoptosis. Consistent with our results in this study, Zheng *et al*. also demonstrated that simvastatin has a protective effect on apoptosis caused by diabetics in bovine retinal capillary endothelial cells and associated with poly (adp-ribose) polymerase activity.[42] Consequently, we proposed that simvastatin ameliorated UVB-induced corneal endothelial cell to apoptosis. However, further evidence is necessary to confirm this observation.

Conclusion

Our results indicate that simvastatin exerts a protective effect against UVB-induced corneal endothelial cell apoptosis through caspase-3 activation.

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Conflicts of interest

There are no conflicts of interest.

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