Inhibition effect of curcumin on UVB-induced secretion of pro-inflammatory cytokines from corneal limbus epithelial cells

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Abstract

• AIM: To study the effects of curcumin on the secretion of interleukin (IL) -6 and IL-8 by corneal limbus epithelial cells.

• METHODS: Human corneal limbus epithelial cells were isolated and cultured from donor eyes and irradiated by UVB at different dosages with or without curcumin. MTT test was used for studying the effects of UVB and curcumin on the cell viability. The role of mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) pathways on the UVB-induced secretion of IL-6 and IL-8 were tested by addition of their inhibitors to the culture with or without UVB-radiation. Levels of various signal pathways, IL-6 and IL-8 in the cells and in the conditioned culture medium were measured by ELISA analysis.

• RESULTS: UVB at 20 mJ/cm² or less and curcumin at 20 μmol/L or less did not affect the cell viability of cultured limbus epithelial cells (P>0.05). UVB irradiation at 10 and 20 mJ/cm² induced a significant increase of secretion of IL-6 and IL-8 and upregulated NF-κB and phosphorylated MAPK pathways of cultured limbus epithelial cells (P<0.05). Various signal pathway inhibitors, including SP600125 (JNK inhibitor), SB203580 (p38 MAPK inhibitor) and BAY11-7082 (NF-κB inhibitor) significantly decreased the UVB-induced secretion of IL-6 and IL-8 secretion (P<0.05).

Curcumin at 5-20 μmol/L significantly inhibited UVB-induced secretion of IL-6 and IL-8 by limbus epithelial cells in a dose-dependent manner; while curcumin alone did not affect the secretion of IL-6 and IL-8. The upregulation of NF-κB and MAPK pathways induced by UVB treatment was significantly inhibited by curcumin, suggesting that NF-κB and MAPK pathways are involved in the inhibitory effect of curcumin on UVB-induced production of IL-6 and IL-8.

• CONCLUSION: Curcumin may be a promising agent to be explored for the prevention and treatment of pterygium.

• KEYWORDS: curcumin; ultraviolet-B; interleukin-6; interleukin-8; corneal limbus epithelial cells; pterygium

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INTRODUCTION

Pterygium is a common ocular surface disease. This is a wing-shaped fibrovascular lesion originated from the limbus and covered by epithelial cells. Pterygium can progress to the center of the cornea and causes loss of vision. Previous studies suggested that pterygium is an inflammatory, invasive, and highly vascularized growths, arise from activated limbus epithelial cells[1-4]. Epidemiological studies demonstrated that chronic exposure to sunlight, especially ultraviolet (UV) irradiation, is the main cause of pterygium. Chronic UV radiation causes the development of pterygium and the recurrence of pterygium after its surgical excision[4-6].
Experimental animal pterygium models have not been established previously. Therefore, an in vitro model has been developed for the investigation of the pathogenesis and treatment of pterygium by using cultured human ocular surface cells from normal tissues or excised pterygium specimens. Pterygium tissue contains a high level of pro-inflammatory cytokines. UVB irradiation on human epithelial cells or fibroblasts isolated from normal ocular surface tissues or surgical excised pterygium specimens stimulate the expression and secretion of several pro-inflammatory cytokines and chemokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-8. This in vitro model has been repeatedly used for studying the pathogenesis of pterygium and for the search of medications that might be used for the prevention and treatment of pterygium.

Chronic inflammatory reaction is involved in the pathogenesis of pterygium. Up-regulation of various pro-inflammatory factors plays an important role in the pathogenesis of pterygium. IL-6 is up-regulated in pterygium tissues. This cytokine has a potent pro-inflammatory effect and also stimulates angiogenesis. IL-8 (CXCL8) attracts neutrophil, T cell and monocytes into the tissues, leads to an inflammatory reaction. IL-8 also induces angiogenesis. All of these effects of these two cytokines lead to the development of inflammatory response and angiogenesis in the pterygium. The expression of IL-6 and IL-8 could be induced by UVB irradiation in normal corneal and pterygium tissues and their various cell components. Pterygium begins growing from limbus epithelial cells and UVB irradiation also induces inflammatory reactions in these cells earlier than other cell types lined ocular surface. Therefore, it is appropriate to use cultured limbus epithelial cells as an in vitro model for the investigation of the effects of UVB and various medication on the progress of pterygium.

Curcumin (diferuloylmethane), is a β-diketones, a yellow coloring agent extracted from turmeric, has a wide array of pharmacological and biological activities including chemopreventive, chemotherapeutic and anti-proliferative potentials. In vitro study, experimental animal study and clinical trials indicated that curcumin can inhibit inflammation via the decrease of expression of various pro-inflammatory cytokines, chemokines, transcription factors and relevant signal pathways. Curcumin inhibits UVB-induced expression of IL-6, IL-8 and TNF-α in keratinocytes through the down-regulation of mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) signal pathways. The effects of curcumin on UVB-induced inflammation in cells from pterygium or normal ocular surface tissues have not been previously reported. The purpose of the present study was to investigate the effects of curcumin on UVB-induced secretion of IL-6 and IL-8 from cultured human limbus epithelial cells and to explore the possibility of using curcumin in the prevention and treatment of pterygium.

MATERIALS AND METHODS

Curcumin Curcumin (99.5% purity) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Curcumin was dissolved in dimethyl sulfoxide (DMSO) to make a 20 mmol/L stock solution and was added to the medium at different concentrations. Cells were treated with 0.25% DMSO as the control group.

Cell Culture Limbus epithelial cells were isolated by us (Hu DN) in the Tissue Culture Center, New York Eye and Ear Infirmary from donor eyes supplied by the New York Eye Bank for Sight Restoration (New York, NY, USA). The Eye Bank obtained the donor’s consent before the collection of the eyes. The principles outlined in the Declaration of Helsinki (2008) have been followed in the present study. The cornea with limbus and 2 mm wide of sclera were excised from the eyeball, then, the cornea and sclera were excised to leave approximately 1 mm on either side of the limbus. The limbus tissue was washed with Hank's solution (GIBCO, Grand Island, NY, USA) three times and than immersed in a 1.2 U/mL dispase II solution (Sigma) for 2h at 37 °C. After the enzymatic dissociation, the limbus epithelial cells were gently scraped by using a iris spatula under the stereo-microscope to isolate the limbus epithelial cells from the Bowman's membrane. Cells were collected and centrifuged. Pellets were resuspended by Ham's F12 nutrient mixture with 10% fetal bovine serum (all from GIBCO), seeded into the culture flask and incubated in a CO2-regulated incubator in humidified 95% air/5% CO2 atmosphere. Seven days later, culture medium were replaced by the defined Keratinocyte-serum free medium (K-SFM, GIBCO). Cells were observed under phase-contrast microscope each day and the culture medium was changed three time a week. After reaching confluence, the limbus epithelial cells were detached using 0.25% trypsin solution (GIBCO), diluted 1:3 and subcultured. Cell cultures in the second passage were used in this study.

Ultraviolet-B Irradiation Limbus epithelial cells were seeded into 12 well plates and grew to 75% confluence. Cultures were washed and covered with a thin layer of PBS before irradiation to remove potential phototoxic materials in culture medium. Cells were irradiated with various dosages of UVB using 20037 312 UVB bulbs (Staratagene, La Jolla, CA, USA), which emit UVB with a spectral peak at 312 nm. PMA 2100 Data Logging Radiometer (Solar Light, Inc., Genside, PA, USA) was used for monitoring and calibrating the intensity of UVB radiation. After UVB irradiation, cells were washed with PBS and cultured with fresh pre-warmed K-SFM for various periods.

MTT Study Cell viability of limbus epithelial cells cultured with or without UVB irradiation was tested by the MTT assay.
Cells at a density of $5\times10^5$ cells/well were seeded onto each well in black well 96-well plates (Sigma). After incubation for 24h, cells were irradiated by UVB at 10, 20 and 50 mJ/cm$^2$ as described above. After 24h, 50 µL tetrazolium bromide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 1 mg/mL) (Sigma) was added and cells were cultured at 37°C. Four hours later, the medium was removed and DMSO (100 µL, Sigma) was added. The optical density as the parameter of cell viability was measured at 540 nm with a microplate reader (Multiskan EX, Thermo, Ventana, Finland). Viability of limbus epithelial cells cultured with or without curcumin was also tested by the MTT assay. Briefly, cells were seeded into 96-well plates. After 24h, cells were cultured with or without curcumin at 10, 20 and 50 µmol/L concentrations for 24h and the cell viability was analyzed by MTT assay as described above.

Effects of Ultraviolet-B and Curcumin on IL-6 and IL-8 Secretion  Limbus epithelial cells were seeded into 12-well plates at the density of 2×10$^5$ cells per well. After cultured for 24h, curcumin at the final concentrations of 5, 10, and 20 µmol/L was added to the culture medium. One hour later, culture medium was withdrawn, washed and cultured with PBS and irradiated with UVB at 20 mJ/cm$^2$ as described above. Immediately after irradiation, PBS was replaced by fresh culture medium. Cells were cultured for 24h. Conditioned culture media were collected and centrifuged to remove dead cells and debris. The supernatants were stored at -70°C until analysis. Tests were performed in triplicate.

Study of p38 Mitogen-activated Protein Kinase and c-Jun N-terminal Kinase Levels in Cultured Limbus Epithelial Cells  Limbus epithelial cells (1×10$^5$) were seeded into 6-well plates. Curcumin was added to the culture to obtain a final concentrations at 20 µmol/L 24h later. Cultures were irradiated with UVB (20 mJ/cm$^2$) 1h later. Cells were harvested 24h later and protein was extracted. c-Jun N-terminal kinase (JNK) and p38 MAPK ELISA kits (with sensitivity at 0.8 U/mL) (Biosource, Camarillo, CA, USA) were used for the measurement of phosphorylated JNK and p38 MAPK levels in protein extracted from collected cells according to the manufacturer’s instructions, respectively. The results (in triplicate) were expressed as percentages of the control, which were the cultures not treated by curcumin and UVB.

Study of NF-κB Levels in Nuclear Extracts from Cultured Limbus Epithelial Cells  Limbus epithelial cells were seeded and treated as described above. Cells were scraped 30min after the treatment of UVB. Nuclear fraction was obtained by the treatment of collected cells with hypotonic buffer (BioSource). Nuclear extracts were obtained by the treatment of nuclear fraction with cell extraction buffer (BioSource). NF-κB ELISA kit (Invitrogen) was used for the measurement of NF-κB levels according to the manufacturer’s instructions. The results (in triplicate) were expressed as percentages of the control, which were the cultures not treated by curcumin and UVB.

Effects of Mitogen-activated Protein Kinase and NF-κB Inhibitors on UVB-induced Secretion of IL-6 and IL-8  Cells (0.2×10$^5$ cells/well) were seeded into multi-well plates (12 well). Signal pathway inhibitors were added 24h later [JNK inhibitor: SP600125; p38 MAPK inhibitor: SB203580; NF-κB inhibitor: BAY11-7082 (Calbiochem, the concentrations of the MAPK inhibitors and NF-κB inhibitor were 10 µmol/L and 5 µmol/L, respectively)] and cultured for 1h. Cells were then treated by UVB as described above. After 24h, conditioned medium was collected, centrifuged and stored at deep freezer.

Measurement of IL-6 and IL-8 Protein Levels  Enzyme-linked immunosorbent assay (ELISA) was used for the measurement of IL-6 and IL-8 protein levels in the supernatant of cultured limbus epithelial cells. Quantikine IL-6 ELISA kit and IL-8 ELISA kit (R&D Systems, Minneapolis, MN, USA) were used to determine the levels of IL-6 or IL-8 according to the manufacturer's instruction. The optical density of the ELISA samples was measured at 450 nm and corrected by 540 nm using a microplate reader (Multiskan EX, Thermo, Ventana, Finland). The amounts of IL-6 and IL-8 (pg/mL) were calculated from a standard curve. The sensitivity of the assay for IL-6 and IL-8 was 0.7 pg/mL and 3.5 pg/mL, respectively. Tests were performed in triplicate.

Statistical Analysis  Each experiment was replicated 3 times and the data were presented as mean±standard deviation (SD). A Student's t-test was performed to assess the significance of difference between the means of the tested group and the controls. Values of $P<0.05$ were considered statistically significant. All data analysis was performed using specific software (SPSS 19.0, SPSS Inc., Chicago, IL, USA).

RESULTS  MTI Study  Cell viability of cultured limbus epithelial cells irradiated by 10-20 mJ/cm$^2$ UVB were not affected by UVB irradiation ($P>0.05$ as compared with the controls). Cells irradiated with UVB at 50 mJ/cm$^2$ showed a significant decrease of cell viability ($P<0.05$) (Figure 1A). Therefore, UVB at 20 mJ/cm$^2$ or less were used in the following experiments. Curcumin at 10-20 µmol/L did not affect the cell viability ($P>0.05$ as compared with the controls). However, viability of cells treated with 50 µmol/L curcumin showed a slight but significant decrease as compared with cells not treated with curcumin ($P<0.05$) (Figure 1B). Therefore, curcumin at 20 µmol/L or less were used in the following experiments.

Effects of UVB on IL-6 and IL-8 Secretion  Cultured human limbus epithelial cells showed relatively low levels of constitutive secretion of IL-6 and IL-8 (Figure 2). UVB irradiation at 5, 10 and 20 mJ/cm$^2$ induced a dose-dependent increase of IL-6 and IL-8 secretion by cultured limbus...
epithelial cells (Figure 2). IL-6 and IL-8 secretion levels in cells irradiated with 10 and 20 mJ/cm$^2$ UVB were significantly greater than those in cells not treated with UVB ($P<0.05$). UVB at 20 mJ/cm$^2$ caused an increase of IL-6 and IL-8 secretion to 5.8- and 4.7-fold of cells not irradiated, respectively (Figure 2).

**Effects of Curcumin on UVB-induced IL-6 and IL-8 Secretion** Curcumin did not affect the constitutive secretion of IL-6 and IL-8 in cultured human limbus epithelial cells (Figure 3). Curcumin at 5, 10 and 20 μmol/L dose-dependently inhibited UVB-induced IL-6 and IL-8 secretion. Both IL-6 and IL-8 secretion in cells treated with UVB and curcumin at 5-20 μmol/L was significantly decreased as compared with the positive controls (cells treated with UVB alone, $P<0.05$) (Figure 3). IL-6 and IL-8 secretion levels in cells treated with UVB and 20 μmol/L curcumin showed no significant difference from those in the negative controls (cells not treated with UVB, $P>0.05$), indicating that curcumin at this dosage can completely block UVB-induced secretion of these two pro-inflammatory cytokines in cultured limbus epithelial cells.

**Effects of Ultraviolet-B on NF-κB and Phosphorylated Mitogen-activated Protein Kinase Levels** UVB treatment induced significant increase of phosphorylated JNK and p38 MAPK levels in limbus epithelial cells ($P<0.05$) (Figure 4). UVB treatment also induced significant increase of NF-κB levels in nuclear extracts of the limbus epithelial cells ($P<0.05$) (Figure 4).

**Effects of Curcumin on UVB-induced Expression of NF-κB and Phosphorylated Mitogen-activated Protein Kinase** UVB-induced increase of phosphorylated JNK and p38 MAPK levels in limbus epithelial cells ($P<0.05$) (Figure 4). UVB treatment also induced significant increase of NF-κB levels in nuclear extracts of the limbus epithelial cells ($P<0.05$) (Figure 4).

**Effects of Mitogen-activated Protein Kinase and NF-κB Inhibitors on UVB-induced IL-6 and IL-8 Secretion** JNK inhibitor, p38 MAPK inhibitor and NF-κB inhibitor significantly decreased the UVB-induced secretion of IL-6 secretion by cultured human limbus epithelial cells ($P<0.05$, as
compared with cells irradiated with 20 mJ/cm\(^2\) UVB) (Figure 5A). Secretion of IL-6 in cells treated with UVB and JNK or p38 MAPK inhibitor still slightly higher than that in cells not treated with UVB (\(P<0.05\)); while secretion of IL-6 in cells treated with UVB and NF-κB inhibitor did not differ from cells not treated with UVB (\(P>0.05\)) (Figure 5A). These results suggested that NF-κB inhibitor can completely block the UVB-induced secretion of IL-6 in limbus epithelial cells, while JNK or p38 MAPK inhibitor only had a partially blocking effect.

**DISCUSSION**

The effects of UVB on the production of IL-6 and IL-8 of limbus epithelial cells have not been reported previously. In the present study, UVB induced significantly increase of secretion of IL-6 and IL-8 by cultured limbus epithelial cells. This is consistent with previously reports, which described the UVB-induced IL-6 and IL-8 by other types of ocular surface epithelial cells, such as normal corneal epithelial cells or pterygium epithelial cells\(^{[7-9]}\).

The signal pathways involved in UVB-induced production of IL-6 and IL-8 by corneal epithelial cells, pterygium epithelial cells and keratinocytes include MAPK and NF-κB.
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Curcumin is a natural anti-inflammatory compound with a long history of use, has been used as a remedy for the treatment and prevention of inflammatory diseases. Aggarwal et al. reported that more than 60 clinical trials had been conducted for studying the efficacy and safety of curcumin. In addition to these studies, another 35 clinical trials have evaluated the efficacy of curcumin. Curcumin was found to be effective in the treatment of various chronic inflammatory diseases including psoriasis, inflammatory bowel diseases and different types of arthritis, such as rheumatoid arthritis and osteoarthritis. It has even been demonstrated to be effective in the treatment of other severe diseases such as Alzheimer’s, cystic fibrosis, and AIDS. Clinical trials also demonstrated that curcumin is a safe, nontoxic remedy, can be given orally and is quite safe and affordable.

The main limitation of curcumin is its insolubility in aqueous systems. Various attempts to enhance the bioavailability and efficacy of curcumin have been reported by the encapsulation of curcumin into liposomes, phospholipid complexes or nanoparticle. The primary aim of these studies is to achieve increased solubilization of curcumin and, at the same time, to protect curcumin against inactivation by hydrolysis. Therefore, in the future, curcumin can be used not only as an oral nutrition supplement, but also can be used locally, such as the eye drops for the treatment of various ocular inflammatory diseases.

The present study found that curcumin at low and safe dosages significantly inhibits the UVB-induced secretion of IL-6 and IL-8 by cultured limbus epithelial cells; but does not affect the constitutive secretion of IL-6 and IL-8. These results suggest that curcumin may be a promising agent to be explored for the prevention of progress of pterygium, and also may be used for the prevention of recurrence of pterygium after its surgical excision.

REFERENCES


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