Effects of Cyclosporin A on Human Conjunctival Fibroblasts

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Objective: To evaluate the effects of cyclosporin A (CsA) on cytokine and/or collagen production, cell growth, and apoptosis in conjunctival fibroblast cultures.

Methods: Fibroblast cultures derived from normal subjects and patients with vernal keratoconjunctivitis and pemphigoid were exposed to different concentrations of CsA for either 24 hours or 30 days. The effects were evaluated by the colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) test to assess cell proliferation, and by the measurement of procollagen I (PIP) and procollagen III (PIIIP) cytokines and total protein in culture medium. CsA-induced apoptosis was assessed by fluorescence-activated cell sorter analysis.

Results: After 24 hours of exposure to doses of CsA of more than 10 µg/mL, cell proliferation and migration were significantly reduced. Cyclosporin A reduced PIP and interleukin 1 (IL-1) production in a dose-dependent manner. Interleukin 6 and IL-8 were increased by 10 µg/mL of CsA, whereas transforming growth factor β, PIIIP, and total protein were unaffected. Cyclosporin A exposure induced apoptosis in a time- and dose-dependent manner. Long-term exposure to CsA reduced IL-6 but did not modify PIIIP production.

Conclusion: Exposure to CsA directly modified fibroblast behavior.

Clinical Relevance: Cyclosporin A ability to accelerate apoptosis in clinically fibrotic tissues may prove to be therapeutic and useful in hyperproliferative conjunctival disorders.


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Clinical trials have suggested that topical cyclosporin A (CsA) is effective and without serious adverse effects for the treatment of corneal graft failure and immune-mediated ocular diseases such as vernal keratoconjunctivitis (VKC), atopik keratoconjunctivitis, Sjögren syndrome, phlyctenular keratoconjunctivitis, ocular pemphigoid, and corneal ulcers. Although these chronic inflammations have different immunopathogeneses, clinical features, and evolution, some of these entities can lead to morphological and functional modifications of the conjunctiva that are associated with different degrees and sites of fibrosis, such as giant papillae, subconjunctival fibrosis, and symblepharon.

Cyclosporine interferes with the antigen-induced phase of T-cell activation, selectively inhibiting gene transcription for interleukin 2 (IL-2), IL-3, IL-4, and interferon γ. Relatively little is known with regard to the effects of CsA on nonhemopoietic cells. It is very hydrophobic and readily diffuses into cell membranes. High and persistent concentrations of CsA have been found in the cornea and sclera after a single application of the 2% ocular solution. Cyclophilin, a cytosolic protein capable of binding CsA, is known to be present in all cell types, including fibroblasts. The CsA-cyclophilin complex has been shown to inhibit the activity of the calcium/calmodulin-dependent phosphatase calcineurin, but not the protein kinase C and A pathways.

There is growing evidence that CsA is also able to affect the biological functions of some nonimmune cell types, including endothelial cells, keratinocytes, skin and gingival fibroblasts, and mast cells. Since CsA is already widely used topically in immune-mediated ocular conditions in which not only T cells but also structural cells are activated, its effect on conjunctival fibroblasts needs to be elucidated. The effect of CsA on fibroblasts has been investigated mostly because of its adverse effect of gingival overgrowth. Cyclosporin A has been shown in vitro and in vivo experiments to either stimulate or inhibit functioning of gingival fibroblasts. Whether these are direct effects of CsA on gingival fibroblast activity or an effect on various cytokines and growth factors produced by inflammatory...
MATERIALS AND METHODS

CELL CULTURES

After informed consent was obtained, biopsy specimens were obtained under topical anesthesia (4% oxybuprocaine eye drops) from the upper tarsal conjunctiva of 4 patients with VKC, from the lower tarsal conjunctiva of 2 patients with pemphigoid, and from the lower tarsal conjunctiva of 4 healthy subjects after subconjunctival injection of 1% me-pivacaine hydrochloride. Clinical research followed the tenets of the Declaration of Helsinki. Biopsy specimens were washed, cut into small pieces, and seeded in Nunc multidishes (NUNC, Roskilde, Denmark) containing 100 µL of Ham’s F12 medium (Sigma, St Louis, Mo), supplemented with 10% fetal calf serum (Sigma) and antibiotics (100 U/mL of penicillin, 100 µg/mL of streptomycin, and 2 mMol/L of L-glutamine; Sigma). Tissues were incubated at 37°C in 5% carbon dioxide in a humidified air atmosphere and fed daily. When cells began to form a monolayer, tissue pieces were removed. Cells were fed with 500 µL of supplemented medium twice a week. When fibroblast cultures reached confluence, they were detached from the wells with trypsin and replated into 24-well plates (>95% vitality). The fibroblasts were characterized morphologically, stained positively with vimentine, and stained negatively with cytokeratines.

EXPERIMENTAL DESIGN

Third- to eighth-passage fibroblasts were used for the experiments. Cyclosporin A was obtained from commercially available 5-mg/mL intravenous preparations (Novartis, Basel, Switzerland) and added to the medium in the following doses: 0, 0.001, 0.01, 0.1, 1.0, 10.0, 50.0, 100.0, or 1000.0 µg/mL. In all the experiments, CsA was diluted with ethanol. Controls in each experiment were cells treated with a medium containing the maximal ethanol concentration used to solubilize and dilute CsA. Before each experiment, culture medium was switched from 10% fetal calf serum to 0.4% fetal calf serum for 24 hours. Then, cells were incubated in medium containing 0.4% serum and different concentrations of CsA. The maximal concentration of CsA (1000 µg/mL) was found to be toxic to cells and was studied no further.

To evaluate the effect of long-term exposure to CsA, fibroblasts at confluence were maintained for 30 days with different concentrations of the drug. Medium supplemented with CsA and 10% serum was changed at day 1, then twice a week and collected for IL-6 and procollagen III (PIIIP) determination at days 1, 3, 15, and 30. Cells were evaluated morphologically by inverted microscope every day and for cell vitality at day 30.

Cyclosporin A concentrations in supernatants were evaluated after 24 hours of culture and found to correlate with expected values in culture medium.

PROLIFERATION ASSAY

To determine the proliferation rate of human conjunctival fibroblasts, the colorimetric tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma] test was performed. Two thousand cells per well were seeded into 96-well tissue-culture plates and then treated with CsA as previously described. After incubation times of 24, 48, and 72 hours, the MTT test was performed. The optical density of each well was measured using an automatic plate reader with a 560-nm test wavelength and a 690-nm reference wavelength. In each experiment, 8 wells were used for the same concentration. Experiments were repeated twice for each fibroblast line.

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RESULTS

EFFECTS OF CSA ON CELL GROWTH AND MIGRATION

Early passage fibroblasts from patients with pemphigoid and patients with VKC showed a higher proliferation rate compared with cells derived from normal subjects. Preliminary effects of CsA on fibroblast proliferation were determined at 24, 48, and 72 hours. The most satisfactory results were obtained with the 24-hour time point since toxic effects were noted at high doses of CsA after 48- and 72-hour incubations. Thus, the subsequent experiments were performed with the 24-hour incubation time.

With increasing doses of as high as 10 µg/mL of CsA per culture, fibroblast growth remained unchanged. Above this concentration, a significant reduction in cell growth was detected compared with that of the minimal concentration of CsA (0.001 µg/mL) (ANOVA, P<.05). The same behavior was observed for all fibroblast lines, both from normal subjects and from those derived from ver- nal and pemphigoid patients. The concentration required for 50% inhibition of cell growth was 61 µg/mL as interpolated from the dose-inhibition curve (Figure 1).

With regard to CsA’s effect on cell migration in the wounded fibroblast monolayer, CsA doses of 10 µg/mL and higher significantly reduced the number of cells behind the wound line when compared with controls (ANOVA, P<.05).

EFFECTS OF CsA ON ANNEXIN V EXPRESSION

Nontreated fibroblast lines cultured in 0.4% fetal calf serum for 24 hours showed a low basal expression of annexin V by fluorescence-activated cell sorter analysis. This expression changed after treatment with CsA for 24 hours in the 3 cell lines derived from pathologic tissues. Annexin V expression increased in a dose-dependent man-
IN VITRO WOUND PRODUCTION MODEL

To assess cell migration, an in vitro wound model was used as previously described.23 Briefly, in confluent cultures in 35-mm dishes, a wound was produced with a 35-mm blade cut. A cotton swab was used to scrape off the fibroblasts from one side of the blade, then the wounded monolayer was washed twice with buffer. After wounding, fibroblast cultures were treated with CsA as described above. Experiments were performed 3 times for each fibroblast population. After incubation, the supernatants were removed, and the wounded cultures were fixed and stained with an ethanol solution of 0.007% toluidine blue with a pH of 3.5 for 1 minute at room temperature. At a magnification of × 400, the total number of fibroblasts located 250 mm beyond the wound line was quantified in at least 5 different fields.

APOPTOSIS-SPECIFIC PROTEIN AND PROPIDIUM IODIDE

To determine any toxic or apoptotic effect of CsA, 12,000 cells per well were seeded into 35-mm dishes and treated with CsA as described. After a 24-hour incubation, cells were removed with trypsin, washed 3 times with phosphate-buffered saline and resuspended in binding buffer. Five microliters of fluorescein isothiocyanate–labeled annexin V (Kamiya, Biomedical Company, Seattle, Wash) were added to cell suspensions, which were then incubated for 10 minutes at room temperature, washed, and resuspended. Ten microliters of propidium iodide stock solution (Becton & Dickinson, San José, Calif) were then added. Fluorescence-activated cell sorter analysis was performed using FACSCalibur (Becton & Dickinson).

EFFECTS OF CsA ON PROCOLLAGENS AND CYTOKINE PRODUCTION

Culture medium of nonstimulated fibroblasts contained detectable amounts of PIP and PIIIP. When CsA was added, PIP levels, expressed in nanograms per microgram of total protein, progressively decreased in a dose-dependent manner (Figure 4). Doses higher than 1 µg/mL of CsA significantly reduced PIP production compared with control levels (ANOVA, P < .05). Production of PIIIP and total protein in culture medium did not change significantly. No significant differences were observed among the different cell lines.

Culture medium of nonstimulated fibroblasts contained detectable amounts of IL-1β, IL-6, IL-8, and TGF-β1. With increasing doses of CsA, IL-1 levels progressively decreased. At the highest doses of CsA (10 µg/mL and 50 µg/mL), IL-1 levels were significantly lower than those found to have the lowest concentration of CsA (0.01 µg/mL). Conversely, the levels of IL-6 and IL-8 significantly increased with 50 µg/mL and 10 µg/mL of CsA, respectively, compared with those found to have the lowest dose of CsA (0.01 µg/mL) (Figure 5). Levels of TGF-β1 did not change significantly.

EFFECTS OF LONG-TERM EXPOSURE TO CsA

Fibroblasts at confluence in 10% serum maintained normal morphology for all 30 days, with concentrations of CsA from 0.001 µg/mL to 10 µg/mL, as well as controls exposed to medium supplemented with ethanol only. Cells treated with 100 µg/mL CsA showed 50% vacuolization after 24 hours of exposure and were completely dead at day 3. Cells exposed to 50 µg/mL of CsA started to decline at day 2, 50% of these demonstrated vacuolization at day 3, and all were completely dead at day 5. On day 30, cell vitality was not affected by doses of CsA ranging from 0.001 µg/mL to 10 µg/mL. Collagen production did not change within 30 days, while IL-6 levels were reduced at day 15 and day 30, compared with levels determined at day 1 (ANOVA, P < .05) with CsA doses of 1 µg/mL and 10 µg/mL.

As a positive control, cells were exposed for 5 minutes to 0.4 mg/mL of mitomycin C, and maintained in 0.4% serum for 24 and 48 hours before analysis.

Time-course experiments were also performed evaluating annexin V expression after 1, 3, 6, 12, and 24 hours of exposure to 10 µg/mL of CsA.

PROCOLLAGEN, TOTAL PROTEIN, AND CYTOKINE PRODUCTION

In the study of CsA’s effect on procollagen synthesis, 6250 cells per well were seeded onto 24-well plates and treated with the same CsA concentrations as described earlier. After an incubation time of 24 hours, the medium was removed and stored at −20°C. Experiments were repeated twice for each fibroblast line. The level of procollagen I (PIP) was determined using an radioimmunoassay method (Orion Diagnostica, Espoo, Finland); PIIIP, using a 2-stage sandwich assay (Cis Bio International, Gif-Sur-Yvette, France); and total protein synthesis, by the Lowry method.

An enzyme-linked immunoassay was used to measure levels of IL-1β, IL-8, transforming growth factor β1 (TGF-β1) (BioSource, Nivelles, Belgium), and IL-6 (DPC, Los Angeles, Calif). Samples were analyzed in duplicate. The sensitivity of the assays was as follow: PIP, 25 pg/mL; PIIIP, 0.1 U/mL; IL-1β, 2 pg/mL; IL-6, 1 pg/mL; IL-8, 1 pg/mL, and TGFβ1, 1 pg/mL.

STATISTICS

Data are presented as the mean ± SD of the percent control values. They were analyzed by analysis of variance (ANOVA) with a post hoc analysis (Fisher protected least significant difference test). Differences among fibroblast populations were calculated using the unpaired t test.
tracted immunomediated keratoconjunctivitis. Cyclosporin A has been used topically as an immunosuppressive agent for its ability to reduce the cytokine expression from T cells, and thus, reduce local inflammation. However, the effects of CsA on resident stromal conjunctival cells and nonhemopoietic cells are less known. Kidney dysfunction and gingival overgrowth are common adverse effects of the systemic use of CsA, probably caused by a direct or indirect effect of the drug on renal proximal tubule cells or renal cortical fibroblasts,\textsuperscript{26} and on gingival fibroblasts.\textsuperscript{18-20} The potential of ocular topical CsA to modify conjunctival fibroblast metabolism, and thus the fibrogenic phases of chronic keratoconjunctivitis, was investigated in this study. Results demonstrated that CsA did influence conjunctival fibroblast metabolism.

Previous pharmacokinetic studies in animals demonstrated persistent levels, from 900 to 1400 ng/mL, in the cornea and sclera after a single application of 2% CsA.\textsuperscript{10} In both dog and rabbit models, single topical doses of CsA in a castor oil–water emulsion formulation reached peak conjunctival concentrations within 1 hour and maintained high concentrations for several hours.\textsuperscript{27} Considering the highly lipophilic characteristics of CsA and its diffusion into cell membranes, the doses used in the present experiments may have closely reproduced in vivo conditions.
Conflicting data have been reported on the effects of CsA in gingival fibroblast cultures. CsA was shown to both stimulate and inhibit PIP, IL-6, collagen I mRNA expression, and cell growth in different gingival cell lines. This discrepancy may have resulted from the use of different and high concentrations of serum. Serum not only contains growth factors that may have muddled results, but also lipoproteins to which CsA nonspecifically binds, thus modifying its availability. In the present study, CsA was shown to have a direct effect on conjunctival fibroblast metabolism by reducing cell proliferation rate and cell migration. Since fibroblasts have been shown to be activated in immunomediated conjunctivitis, inducing both tissue remodeling and scarring, these effects of CsA may have relevant clinical implications for the down-regulation of conjunctival structural cells. These data agree with a previous finding of CsA having inhibited rabbit subconjunctival fibroblast proliferation.

Clinical and histopathological benefits of local CsA administration have been suggested in several immunomediated corneal and conjunctival diseases. The immunosuppressive effect of CsA may be exerted through inhibition of cytokine and mitogen-induced gene expression. In vitro studies have established that CsA affects the initial mitogen- or antigen-induced phase of T-cell activation, selectively inhibiting the induction of a small number of genes responsible for IL-2, IL-3, IL-4, INF-γ and c-Myc, and several mitogen-induced genes. In the present study, all conjunctival cell lines spontaneously produced and released detectable amounts of IL-1β, IL-6, IL-8, TGF-β1, PIP, and PIIP. Exposure to CsA for 24 hours reduced the production of IL-1β and procollagen I in a dose-dependent manner starting from 1 µg/mL of CsA. Interleukin 6 and IL-8 concentrations were unchanged with CsA exposures of up to 1 µg/mL, then were significantly increased with doses of CsA ranging from 10 µg/mL to 50 µg/mL. This may be related simply to the toxic activity of high doses of CsA, and it may explain the irritation reported by some patients treated with topical CsA. This finding is in agreement with the reported up-regulation of IL-6.
expression by gingival fibroblasts exposed to CsA. However, in cultures exposed to 1 µg/mL and 10 µg/mL of CsA for 30 days, IL-6 production was reduced without affecting cell vitality and collagen production, confirming that doses similar to those obtained with the current clinical use, can modulate fibroblast activity.

Cyclosporin A has been shown to either promote or inhibit apoptosis in a dose-dependent manner in different experimental models and cell lines. Apoptosis can be inhibited by cyclosporin A in a dose-dependent manner in different assays and cell lines.31-35 Apoptosis can be triggered in conjunctival fibroblasts by topically applied cytotoxic drugs such as mitomycin C. In dogs affected by chronic idiopathic keratoconjunctivitis sicca, 0.2% topical CsA was shown to stimulate infiltrating lymphocyte apoptosis and reduce epithelial cell apoptosis, suggesting that CsA facilitates the reestablishment of a normal apoptotic equilibrium. The induction or suppression of apoptosis in different diseases and cells may depend on the cell cycle phase, cell type, or the presence of other factors. In the present study, CsA’s induction of apoptosis in 3 cell lines derived from pathologic tissues with signs of scarring lends support to the therapeutic use of this drug in hyperproliferative conjunctival disorders. Interestingly, the CsA concentration that stimulated apoptosis was identical to that shown to inhibit cell proliferation and increase IL-6 and IL-8 release. Toxic effects of CsA, shown by increased propidium iodide captation, were found with doses equal to or greater than 50 µg/mL.

Studies using tissues from patients with pemphigoid and VKC reported both an increased expression of fibrogenic cytokines and growth factors.37,38 and an increased proliferation rate of cultured primary fibroblasts.28-32 The successful use of antimetabolite agents for the management of pemphigoid34 and severe VKC35 provides additional evidence that apoptosis is involved. Long-term management of VKC with topical CsA is known to reduce conjunctival inflammation and the size of limbal infiltrates and giant papillae. In the past, this last effect was considered secondary to the immunosuppressive activity of CsA. However, the present study provides evidence for the immunosuppressive activity of CsA in patients with secondary Sjögren’s syndrome.

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