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-di-methylthiazol-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.


CLINICAL TRIALS have suggested that topical cyclosporin A (CsA) is effective and without serious adverse effects for the treatment of corneal graft failure and immunemediated ocular diseases such as vernal keratoconjunctivitis (VKC), atopic 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 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% mepivacaine hydrochloride. Clinical research followed the tenets of the Declaration of Helsinki. Biopsy specimens were washed, cut into small pieces, and seeded in Nunclon Multidishees (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 cells remains to be clarified. A recent study showed that CsA reduced rabbit subconjunctival fibroblast proliferation in a dose-dependent manner, indicating an antiproliferative effect of this drug. Induction or modulation of apoptosis may be one of the mechanisms of action of CsA on different cells. Fibroblast apoptosis may be induced by nutritional and cytokine deprivation and by exposure to antimitabolite agents such as mitomycin C.

The aim of the present study was to evaluate in vitro the direct effects of clinically relevant doses of CsA on conjunctival fibroblast functions by assessing cell migration and proliferation, the production of procollagens and cytokines, and the induction of apoptosis.

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 vernal 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. 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 µm 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 FACS Calibur (Becton & Dickinson).

Fibroblast overgrowth, extracellular matrix deposition, and fibrosis are some of the consequences of severe and pro-
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,26 and on gingival fibroblasts.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.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.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\textsuperscript{18,19} 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,\textsuperscript{28,29} these effects of CsA may have relevant clinical implications for the downregulation of conjunctival structural cells. These data agree with a previous finding of CsA having inhibited rabbit subconjunctival fibroblast proliferation.\textsuperscript{22}

Clinical\textsuperscript{1-8} and histopathological\textsuperscript{30,31} 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-\gamma and c-Myc, and several mitogen-induced genes.\textsuperscript{9,32} In the present study, all conjunctival cell lines spontaneously produced and released detectable amounts of IL-1\beta, IL-6, IL-8, TGF-\beta1, PIP, and PIHP. Exposure to CsA for 24 hours reduced the production of IL-1\beta and procollagen I in a dose-dependent manner starting from 1 \mu g/mL of CsA. Interleukin 6 and IL-8 concentrations were unchanged with CsA exposures of up to 1 \mu g/mL, then were significantly increased with doses of CsA ranging from 10 \mu g/mL to 50 \mu g/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

\textbf{Figure 5.} Effect of cyclosporin A (CsA) on basal production of cytokines in fibroblast cultures in vitro. Data are expressed as mean percent control levels. A 24-hour exposure to CsA reduced in a dose-dependent manner the production of interleukin 1\beta (IL-1\beta) (A). Interleukin 6 (IL-6) (B) and IL-8 (C) were significantly increased. D, Transforming growth factor \beta1 (TGF-\beta1) production was unaffected by CsA. Asterisks indicate \(P<0.05\) compared with the lowest concentration of CsA using analysis of variance.
expression by gingival fibroblasts exposed to CsA. 18 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 viability 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. 33-35 Apoptosis can be triggered in conjunctival fibroblasts by topically applied cytotoxic drugs such as mitomycin C. 21 In dogs affected by chronic idiopathic keratoconjunctivitis sicca, 0.2% topical CsA was shown to stimulate infiltrating lymphocyte apoptosis and reduce epithelial cell apoptosis, 36 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. 28,29 The successfull use of antimetabolite agents for the management of pemphigoid 39 and severe VKC 40 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. From the present study, within the limits of in vivo and in vitro comparisons, topical CsA may be considered not only an immunosuppressive agent, but also a direct fibroblast inhibitor that may also promote apoptosis.

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