Cyclosporin A Inhibits Calcineurin/Nuclear Factor of Activated T-Cells Signaling and Induces Apoptosis in Retinoblastoma Cells

Lauren A. Eckstein,¹ Kurtis R. Van Quill,¹ Steven K. Bui,² Marita S. Uusitalo,³ and Joan M. O'Brien¹

PURPOSE. Although the clinical efficacy of cyclosporin A (CSA) in retinoblastoma (RB) has been attributed to multidrug resistance reversal activity, the authors hypothesized that CSA is also directly toxic to RB cells through inhibition of calcineurin (CN)/nuclear factor of activated T-cells (NFAT) signaling.

METHODS. Antiproliferative effects of CSA, PSC-835 (a CSA analogue that does not inhibit CN), and FK506 (a CN inhibitor structurally unrelated to CSA) were evaluated in Y79 and Weri-RB1 cells by WST-1 assay. Apoptosis induction by CSA and PSC-835 was measured by detection of caspase 3/7 activity and by flow cytometry, using annexin-V and 7-AAD stains. Expression of CN was assayed in RB cells by immunocytochemistry. Expression of NFAT, a CN-dependent transcription factor family, and FK506 binding protein 12/12.6 (FKBP12/12.6), effectors of CN inhibition by FK506, was assayed in RB cells by Western blot analysis. NFAT activity was assayed in CSA-treated and -untreated Y79 cells transfected with an NFAT-sensitive reporter gene.

RESULTS. CSA induced dose-dependent antiproliferative and proapoptotic effects at clinically achievable levels in Y79 and Weri-RB1 cells. PSC-835 induced antiproliferative effects only at nonphysiologic concentrations with minimal associated apoptosis. FK506 induced minimal antiproliferative effects in RB cell lines, probably due to trace or absent FKBP12/12.6 expression. RB cell lines expressed CN-α, CN-β, NFATc1, and NFATc3. CSA treatment also potently inhibited NFAT-mediated reporter gene transcription.

CONCLUSIONS. These results demonstrate functional integrity of the CN/NFAT signaling cascade in RB cells and suggest that CSA is cytotoxic to RB cells through inhibition of this pathway and consequent apoptosis induction. (Invest Ophthalmol Vis Sci. 2005;46:782-790) DOI:10.1167/iovs.04-1022

Retinoblastoma (RB) is the most common ocular cancer in children, representing 12% of all infant cancers.¹ These neoplasms arise from loss or mutation of both alleles of the RB tumor suppressor gene (RB1) in the developing retina.²,³ In nonheritable RB, both RB1 alleles are inactivated somatically in a single retinoblast. In heritable RB, one allele is mutated in the germline, and loss of the second allele occurs in developing retinal cells, typically resulting in multifocal, bilateral disease.

Larger RB tumors have traditionally been treated with enucleation or with external beam radiation therapy (EBRT). Although EBRT is often curative, it produces midface bony deformities and increases second tumor risk in patients with heritable disease.⁴ Recognition of these risks and side-effects has prompted interest in chemotherapy as an alternative to EBRT for the treatment of intraocular disease.

Empiric trials have demonstrated that chemotherapy is effective in the treatment of intraocular RB.⁵-⁹ Chemoreduction with carboplatin, etoposide, and vincristine (CEV) plus adjuvant local treatment is now a first-line therapy for ocular salvage in RB, with EBRT reserved for patients with advanced disease at presentation or chemoresistant disease. Promising results have also been reported with high-dose CSA, an immunosuppressant administered as an adjuvant to CEV in advanced or poorly responsive disease.¹⁰,¹¹

CSA was introduced in RB with the rationale that it would inhibit P-glycoprotein (Pgp)-mediated multidrug resistance (MDR).¹⁰ Pgp is a membrane-bound efflux pump that transports toxins and certain chemotherapeutic agents, including etoposide and vincristine, out of cells.¹² In many tumors, including RB, increased Pgp expression has been correlated with chemotherapeutic resistance.¹²,¹³ By inhibiting Pgp, CSA has been shown to reverse Pgp-mediated MDR and to increase intracellular concentrations of chemotherapeutic agents in vitro.¹⁴ Although carboplatin is not a substrate of Pgp, CSA may also sensitize tumor cells to platinum therapy by inhibiting platinum-induced oncogene expression.¹⁵,¹⁶ We hypothesized that the therapeutic effects of CSA in RB could also be mediated, in part, by direct cytotoxicity. Neurotoxicities associated with CSA therapy¹⁷,¹⁸ suggest that CSA may also be toxic to RB cells, which are derived from neuronal precursors capable of both neuronal and glial differentiation.¹⁹ Consistent with this view, an early report demonstrated that CSA exerts antiproliferative effects in Y79 and Weri-RB1 RB cell lines at clinically achievable concentrations.²⁰

Clues to the mechanism of CSA's antiproliferative effects in RB cells are provided by in vitro studies in related cell types. CSA induces apoptosis in mixed murine cortical and rat hippocampal cultures,²¹,²² and in rat C6 glioma cells.²³ In C6 cells, apoptosis induction by CSA is correlated with inhibition of

From the ¹Ocular Oncology Unit, Department of Ophthalmology, University of California at San Francisco, San Francisco, California; the ²Department of Ophthalmology, University of California at Irvine, Irvine, California; and the ³Department of Ophthalmology, Helsinki University Central Hospital, Helsinki, Finland.

Supported in part by That Man May See, Inc. (San Francisco, CA); the Sand Hill Foundation (Menlo Park, CA); a Physician Scientist Award from Research to Prevent Blindness (JMO); and Grant EY15812 (JMO) and Core Grant EY02162 from the National Eye Institute, Bethesda, Maryland. L.A. Eckstein was supported by a Postdoctoral Research Grant from Knights Templar Eye Foundation, Inc. (Chicago, IL), and by a Postdoctoral Fellowship from Fight For Sight, Inc. (Shaumburg, IL). MSU was supported by the Mary and Georg C. Ehrnrooth Foundation (Helsinki, Finland); The Finnish Eye Foundation, Helsinki, Finland; and the Academy of Finland.

Submitted for publication August 25, 2004; revised November 15, 2004; accepted November 22, 2004.

Disclosure: L.A. Eckstein, Barnet Products Corp. (F); K.R. Van Quill, None; S.K. Bui, None; M.S. Uusitalo, None; J.M. O’Brien, Novartis Pharma AG (F)

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Joan M. O’Brien, Director, Ocular Oncology Unit, UCSF Department of Ophthalmology, 10 Koret Way, Box 0730, San Francisco, CA 94143; aleja@itsa.ucsf.edu.


Copyright © Association for Research in Vision and Ophthalmology

782
CN/NFAT signaling, the principle mechanism of action for this agent.\textsuperscript{24} CN is a signaling intermediary expressed in many cell types,\textsuperscript{25} including RB cells.\textsuperscript{26} CSA inhibits CN by binding first to the ubiquitous cytosolic protein, cyclophilin. The CSA/cyclophilin complex then binds and inhibits the enzymatic region of CN.\textsuperscript{27} The best-described substrates of CN are the NFAT family transcription factors. NFAT proteins were first identified in T-cells,\textsuperscript{28} where they mediate the immune response. In T-cells, antigen presentation at the cell surface initiates a signaling cascade that activates CN, which in turn dephosphorylates cytosolic NFAT. This action induces translocation of NFAT to the nucleus, where it activates transcription of target genes, including interleukin-2, a central mediator of immune activation and T-cell proliferation. CSA induces immunosuppression by inhibiting this pathway.\textsuperscript{27} NFAT proteins have since been detected in other cell types, where they regulate diverse cellular processes, including growth, differentiation, and development.\textsuperscript{29–31} Five isoforms have been described, including NFAT types c1 to c4,\textsuperscript{32–36} and NFATc5, a variant family member that does not interact with CN.\textsuperscript{37}

With these considerations in mind, we hypothesized that CN-mediated, NFAT-dependent signaling also exists in RB cells and that the antiproliferative effects of CSA in these cells result from inhibition of CN/NFAT signaling with consequent induction of apoptosis. To explore this hypothesis, we first sought to confirm the previously described antiproliferative effects of CSA in RB cells and to determine whether these effects are mediated by induction of apoptosis. We also tested the role of CN inhibition in the action of CSA by comparing the antiproliferative and proapoptotic effects of CSA with those of PSC-833 and FK506 in RB cells. PSC-833 is a structural analogue of CSA that potently inhibits Pgp, but does not inhibit CN.\textsuperscript{38–40} In contrast, FK506 is an immunosuppressive agent structurally unrelated to CSA that inhibits CN 10 times more potently than CSA.\textsuperscript{41} Finally, we confirmed that CN and NFAT are expressed in RB cells, and examined the functionality and CSA-sensitivity of the CN/NFAT-signaling pathway in these cells.

### Materials and Methods

#### Cell Lines

Jurkat human T-cells and Y79 and Weri-RB1 human RB cell lines were maintained in RPMI-1640 medium supplemented with 15% fetal bovine serum (FBS), penicillin, and streptomycin.

#### Determination of Dose-Dependent Antiproliferative Effects of CSA, PSC-833, and FK506

Cells were seeded into 96-well microtiter plates and treated with CSA (Novartis Pharma AG, Basel, Switzerland), PSC-833 (a gift from Novartis Pharma AG), FK506 (Fujisawa Healthcare, Inc., Deerfield, IL), or dose-matched vehicle controls. CSA and PSC-833 were provided in cremophor EL (Sigma, St. Louis, MO) and ethanol; FK506 was provided in polyoxyl 60 hydrogenated castor oil (a gift from Barnet Products Corp., Englewood Cliffs, NJ) and ethanol. After incubation, cell viability was determined by assay (WST-1 Cell Proliferation Assay; Roche Diagnostics, Mannheim, Germany) per the manufacturer’s instructions.

#### Quantification of Apoptosis by Caspase Activity Detection and Annexin-V/7-AAD Staining

Y79 cells were treated with CSA, PSC-833, or vehicle control as described earlier. After incubation, caspase activity was quantified by a luminescence assay (Caspase-Glo 3/7 Assay; Promega, Madison, WI) per the manufacturer’s instructions. For annexin-V/7-AAD staining, treated cells were labeled with annexin-V (APC-conjugated, 1:50 dilution; BD PharMingen, San Diego, CA) and 7-AAD (BD PharMingen) in annexin-V binding buffer (BD ApoAlert; BD Clonetech, Palo Alto, CA) per the manufacturer’s instructions. Cells (1 × 10\(^6\) per sample) were counted by flow cytometry (FACSCalibur flow cytometer; BD, Franklin Lakes, NJ), and data were analyzed on computer (FlowJo software; TreeStar, San Carlos, CA).

### Immunohistochemical Detection of CN

Y79 and Jurkat cells were plated overnight on poly-\(\gamma\)-lysine-coated chamber slides (BD Labware, Bedford, MA). Next, cells were serially fixed with 4% paraformaldehyde and with −20°C methanol and then blocked in normal horse serum (1:50 dilution in PBS with 2% BSA; Vector, Burlingame, CA). Cells were then labeled with mouse monoclonal antibodies recognizing the α- or β-subunit of CN (clones CN-A1 and CN-B1, respectively, 1:4 dilution in PBS/2% BSA; Sigma-Aldrich), biotinylated anti-mouse secondary antibody (1:200 dilution in PBS/2% BSA; Vector), and Texas red avidin D (1:200 dilution in PBS; Vector). Slides were photographed with a fluorescence microscope at 450 to 490 nm. Negative controls were prepared by excluding primary antibody.

### Western Blot Analysis of NFAT Family Proteins in RB Cells

Whole-cell extracts of Jurkat and Y79 cells were prepared by lysis in RIPA buffer, sonic disruption, and centrifugation at 10,000g to pellet debris. For NFATc1 and NFATc3, 500 µg of total protein was diluted in PBS containing 10 µg of agarose-conjugated anti-NFATc1 or anti-NFATc3 mouse monoclonal antibodies (clones 7A6 and F-1, respectively; Santa Cruz Biotechnology, Santa Cruz, CA). For NFATc2 and NFATc4, 500 µg of total protein was diluted in PBS containing 2 µg of anti-NFATc2 (H66-G5 mouse monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-NFATc4 (H-74 rabbit polyclonal or C-20 goat polyclonal; Santa Cruz Biotechnology) antibodies and 20 µL of appropriate agarose-conjugated anti-isotype antibodies (Santa Cruz Biotechnology). Immunoprecipitate reactions were incubated overnight with gentle shaking at 4°C. Agarose beads were then pelleted, washed with RIPA buffer, resuspended in gel loading buffer, and briefly heated in boiling water. The solution was then subjected to standard SDS-PAGE and Western blot analysis with primary antibodies recognizing NFATc1, -c2, -c3, or -c4 (Santa Cruz Biotechnology); biotinylated anti-mouse, anti-rabbit, or anti-goat secondary antibodies (Vector); and streptavidin-horseradish peroxidase conjugate (Amersham, Buckinghamshire, UK), with visualization by chemiluminescent reaction (ECL-Plus System; Amersham) per the manufacturer’s instructions. Negative controls were prepared by excluding primary antibody from the Western blot analysis protocol.

#### Assessment of NFAT-Mediated Signaling in Y79 Cells

Y79 or Jurkat control cells (2 × 10\(^6\)) were cotransfected with DNA plasmids (a generous gift from Arthur Weiss, MD, PhD, University of California at San Francisco, San Francisco, CA) encoding a constitutively active β-gal expression vector and an NFAT-sensitive luciferase reporter gene\textsuperscript{42–43} by cell transfection reagent (DMRIE-C; Invitrogen, Carlsbad, CA) in reduced-serum medium (Opti-MEM; Invitrogen) per the manufacturer’s protocol. Cells were subsequently maintained in RPMI-1640 medium without phenol red (Invitrogen) and with 15% FBS. At 30 or 47 hours after transfection, cells were treated with 50 µg/mL CSA or with vehicle control. At 48 hours after transfection, cultures were treated with ionomycin (10 µg/mL; Sigma-Aldrich) and phorbol 12-myristate 13-acetate (PMA, 0.25 µg/mL; Sigma-Aldrich), or with vehicle control. At 51 hours post-transfection, luciferase activity was quantified by a luminescence assay (Luciferase Reporter Gene Assay, Constant Light Signal; Roche) per the manufacturer’s instructions. β-gal expression was measured by a luminescence assay (Ga-
Western Blot Analysis of FKBP12/12.6 Expression in RB Cells

Total protein (35 µg) from whole cell extracts of Jurkat, Y79, and Weri-RB1 cells was subjected to standard SDS-PAGE and Western blot analysis using antibodies recognizing actin (C-2 mouse monoclonal, 1:200 dilution; Santa Cruz Biotechnology), CN α-subunit (CN-A1 mouse monoclonal, 1:2000 dilution; Sigma-Aldrich), and FKBP12/12.6 (clone N-19 goat polyclonal, 1:500 dilution; Santa Cruz Biotechnology), biotinylated anti-mouse and anti-goat secondary antibodies (Vector), and streptavidin-horseradish peroxidase conjugate (Amersham) with visualization by chemiluminescent reaction (ECL-Plus System, Amersham).

RESULTS

Antiproliferative Effects of CSA in Y79 Cells

To determine whether CSA exerts direct antiproliferative effects in RB cells, we measured cell titers in Y79 cell cultures treated with up to 100 µg/ml CSA for 24, 48, 72, or 96 hours. These studies demonstrated a dose-dependent reduction in cell counts of CSA-treated cells relative to dose-matched vehicle control cultures (Fig. 1A).

At 24 hours after treatment with CSA, cell viability was only minimally reduced at clinically observed concentrations, which approach 30 µg/ml in plasma of patients with RB receiving high-dose CSA. Significant reductions in cell viability were observed only at the highest concentrations tested. At later time points, however, dramatic reductions in cell viability were observed at clinically achievable concentrations. At 48 hours after treatment, sharp declines in viability were evident at concentrations exceeding 20 µg/ml. Similar dose-response curves were observed at 72 and 96 hours, with even sharper decreases in cell viability at doses exceeding 20 µg/ml. This decline in viability was most pronounced at 96 hours, when no reduction in viability was detected in cells treated with 20 µg/ml CSA, but viability was reduced to just 17% of control at 30 µg/ml (Fig. 1A).

Antiproliferative Effects of PSC-833 and FK506 in RB Cells Compared with CSA

To test the hypothesis that CSA exerts antiproliferative effects in RB cells through inhibition of CN, we compared the effects of CSA, PSC-833, and FK506 in Y79 and Weri-RB1 cell lines. We predicted that if the antiproliferative effects of CSA in RB cells were mediated by inhibition of CN, then PSC-833, a CSA analogue that does not inhibit CN but does inhibit Pgp, would demonstrate reduced antiproliferative effects compared with CSA. We also predicted that FK506, a structurally unrelated immunosuppressant that is a more potent inhibitor of CN than CSA, would demonstrate more potent antiproliferative effects than CSA at equimolar concentrations. For these experiments, we measured cell titers in Y79 or Weri-RB1 cultures treated with up to 100 µg/ml CSA, PSC-833, or FK506 for 96 hours.

As we predicted, the antiproliferative effects of PSC-833 were much less potent that those of CSA (Figs.1B, 1C). This difference in dose response suggests that CSA and PSC-833 exert their antiproliferative effects through different mechanisms of action. As both PSC-833 and CSA inhibit Pgp, but only CSA inhibits CN, we suggest that the additional antiproliferative effect associated with CSA is probably due to inhibition of CN by this agent.

Unexpectedly, FK506 demonstrated little or no antiproliferative effect in RB cells. In Y79 cultures, FK506 reduced cell titers relative to control only at the highest concentrations tested (Fig. 1B), whereas in Weri-RB1 cells, FK506 induced no reduction in viability relative to control, even at the highest concentration tested (Fig. 1C). These data initially appeared to contradict our hypothesis that CSA exerts its antiproliferative effect in RB cells by inhibition of CN, since FK506 would be expected to inhibit CN potently and thereby to inhibit RB cellular proliferation.

Expression of FKBP12/12.6 in RB Cells

We sought to resolve the contradiction between our hypothesis and the results of our FK506 studies by determining whether the required effectors of CN inhibition by FK506 are present in RB cells. Like CSA, FK506 must bind to an intracellular immunophilin protein receptor before it is capable of inhibiting CN. Whereas CSA binds to members of the cyclo-
To continue our investigation of the mechanism of action of CSA in Y79 cells we sought to determine whether CSA’s antiproliferative action is due to minimal or absent FKBP12/12.6 expression relative to CN within T cells accounts for the exquisite sensitivity of these cells to the actions of CSA and FK506. We hypothesized that the limited antiproliferative action of FK506 in RB cells could be due to minimal or absent FKBP12/12.6 expression relative to CN’s expression in these cells.

To evaluate the relative expression of CN and FKBP12/12.6 in RB cells, whole cell lysates of Y79, Weri-RB1, and Jurkat cultures (positive control) were subjected to Western blot analysis with antibodies recognizing CN, FKBP12/12.6, and actin (included to control for differences in protein loading between lanes). Roughly equivalent expression of CN was observed in Jurkat and Y79 cells, and much higher levels of CN expression were detected in Weri-RB1 cells (Fig. 2). Whereas we detected a distinct band corresponding to FKBP12/12.6 in Jurkat cells, only trace quantities of FKBP12/12.6 were detectable in Y79 cells, and no FKBP12/12.6 expression was detectable in Weri-RB1 cells (Fig. 2). These results demonstrate a stoichiometric excess of CN relative to FKBP in RB cells. Minimal detectable expression of FKBP provides a parsimonious explanation for the insensitivity of RB cells to FK506. We conclude that the modest antiproliferative effects of FK506 in Y79 cells may be attributable to trace expression of FKBP12/12.6 in these cells, whereas the ineffectiveness of FK506 in Weri-RB1 cells is probably the result of absent FKBP12/12.6 expression.

Apoptotic Effects of CSA in Y79 Cells

To continue our investigation of the mechanism of action of CSA in Y79 cells we sought to determine whether CSA’s antiproliferative effects are mediated by induction of apoptosis. We began by using a luminescence assay to detect the activity of caspases 3 and 7, markers for apoptotic but not necrotic cells, in Y79 cells treated with 35 μg/mL CSA for 24, 48, 72, or 96 hours. We detected only modest caspase activity at 24 and 48 hours (Fig. 3A), consistent with the more limited reduction in cell titer seen in CSA-treated Y79 cell cultures at these early time points (Fig. 1A). However, at 72 hours, we detected a nearly sevenfold induction of caspase activity relative to vehicle-treated control cells, suggesting that CSA inhibits Y79 cell proliferation through induction of apoptosis. Caspase activity at 96 hours was slightly reduced compared with activity at 72 hours, suggesting that caspase activation may peak at ~72 hours after treatment. This is consistent with the dramatic reduction in cell viability observed at this time point (Fig. 1A).

To confirm that CSA induces apoptosis in Y79 cells, we next measured the frequency of apoptotic cells in CSA-treated cultures by annexin-V and 7-AAD staining with flow cytometry analysis. Annexin-V binds phosphatidyl serine within the plasma membrane of both apoptotic and necrotic cells, whereas 7-AAD is a vital dye that is excluded by apoptotic cells but is absorbed by necrotic cells. These staining characteristics allow identification of apoptotic cells by virtue of staining with annexin-V and exclusion of 7-AAD, and identification of necrotic cells by virtue of staining with both reagents. As in the previous experiment, Y79 cells were treated with 35 μg/mL CSA for 24, 48, 72, or 96 hours.

These studies confirmed that a significant fraction of CSA-treated cells was undergoing apoptosis at 48, 72, and 96 hours. The observed frequency of apoptotic cells increased over time, reaching a peak at 96 hours (Fig. 3B), at which point most cells remaining in culture were either apoptotic or necrotic. At 72 hours, the frequencies of apoptotic and necrotic cells were 49% ± 10% (mean ± SD) and 11% ± 2.7%, respectively, and at 96 hours, 56% ± 6.2% and 21% ± 10%, respectively (Fig. 3B and data not shown). In contrast, in vehicle-treated control cultures, <6% of cells were undergoing apoptosis and <1.5% of cells were undergoing necrosis at any time point examined (Fig. 3B and data not shown). These results provide independent confirmation of our earlier studies of caspase activation and support the conclusion that CSA inhibits Y79 cell proliferation through induction of apoptosis and, to a lesser degree, necrosis.

Apoptotic Effects of PSC-833 in Y79 Cells Compared with CSA

To test the hypothesis that CSA induces apoptosis in RB cells though inhibition of CN, we next compared the degree of apoptosis induced by CSA and PSC-833 in Y79 cells at their 50% inhibitory concentrations (IC50s), as determined by our comparative studies of these agents in Y79 cells (35 μg/mL for CSA and 60 μg/mL for PSC-833; Fig. 1B). At 72 hours after treatment, we observed a sevenfold induction of caspase activity relative to control in cultures treated with CSA at 35 μg/mL, but only minimal induction of caspase activity in cultures treated with PSC-833 at the same concentration (Fig. 3C). This result was consistent with the modest antiproliferative effect induced by PSC-833 at this concentration (Fig. 1B). When cells were treated with 60 μg/mL CSA, we observed an even larger (ninefold) increase in caspase activity relative to the control, whereas treatment with 60 μg/mL PSC-833 resulted in only a twofold induction of caspase activity (Fig. 3C). These results suggest that at their respective IC50s, PSC-833 induces significantly less apoptosis in Y79 cells than does CSA and that the antiproliferative effect of PSC-833 may instead be mediated by induction of necrosis or by cytostatic effects.

In addition, annexin-V and 7-AAD staining showed that in cultures treated for 72 hours with 35 μg/mL CSA, nearly 50% of cells were undergoing apoptosis and <12% of cells were undergoing necrosis. In cultures treated with 35 μg/mL PSC-833, 15% of cells were undergoing apoptosis and <2% of cells were undergoing necrosis (Fig. 3D and data not shown). In cultures...
treated with 60 μg/mL CSA, 74% of cells were undergoing apoptosis, and the remainder were undergoing necrosis. By comparison, in cultures treated with 60 μg/mL PSC-833, just 19% of cells were undergoing apoptosis, and <4% were undergoing necrosis (Fig. 3D and data not shown). These results indicate that in contrast to CSA, PSC-833 induces only modest apoptosis in Y79 cells, even at high concentrations. Moreover, the absence of significant necrosis in these cultures suggests that the antiproliferative effects of PSC-833 may be predominantly cytostatic. These data confirm the results of our caspase studies and provide further support for our conclusion that CSA mediates apoptosis in RB cells through inhibition of CN.

Expression of Calcineurin and Members of the NFAT Family of Transcription Factors in Y79 Cells

To support the hypothesis that CSA induces apoptosis in RB cells through direct inhibition of CN and consequent disruption of NFAT-mediated signaling, we next sought to demonstrate that CN and NFAT are expressed in RB cells. We began by staining whole-cell mounts of Y79 cells with antibodies recognizing either the α or β subunits of CN. These studies revealed robust expression of both protein subunits in the cytoplasm and in the nucleus of Y79 cells (Figs. 4A, 4B). The classic rosette morphology of RB cells was also observed. As a positive control, we stained the Jurkat T-cell line with the same antibodies, demonstrating significant expression of both CN subunits in this cell type (Figs. 4C, 4D). Exclusion of the primary antibody from the staining protocol resulted in minimal background staining (data not shown).

We next sought to determine whether members of the NFAT family of transcription factors are expressed within Y79 cells. Because NFATc5 does not complex with CN and thus does not participate in CN-mediated signal transduction, we focused our investigations on NFATc1, -c2, -c3, and -c4. Whole-cell lysates of Y79 and Jurkat cultures (positive control) were subjected to immunoprecipitation and Western blot analysis with antibodies recognizing NFATc1, -c2, -c3, or -c4. Examination of NFATc1 expression revealed three identical bands in Y79- and Jurkat-derived samples (Fig. 5A). The appar-
transfection. Group 2 was treated with 50 Μg/mL CSA at 47 hours after transfection and stimulated with ionomycin and PMA at 48 hours after transfection (i.e., after 1 hour of preincubation with CSA). Group 3 was treated with 50 Μg/mL CSA at 30 hours after transfection and stimulated with ionomycin and PMA at 48 hours after transfection (i.e., after 18 hours of preincubation with CSA). Control cultures were treated with vehicle alone. After 3 hours of stimulation with ionomycin and PMA (at 51 hours after transfection) cells were lysed, and the luciferase activity was quantified by luminescence assay.

These experiments demonstrated a high endogenous luciferase signal in untreated Y79 cultures, which was only minimally augmented by addition of ionomycin and PMA to the culture media (Fig. 6, first bar), indicating that NFAT is constitutively active in these cells. Pretreatment of Y79 cultures with CSA 1 hour before stimulation with ionomycin and PMA abrogated the stimulatory effect of these agents and also resulted in a 30% reduction in luciferase signal intensity compared to vehicle-treated controls (Fig. 6, second bar). Longer incubation with CSA, for 18 hours before treatment with ionomycin and PMA, resulted in a 94% reduction in luciferase activity (Fig. 6, third bar). No significant reduction in β-gal activity was observed in cells pretreated with CSA, indicating that the attenuation of luciferase signal could not be attributed simply to cytosstatic or cytoidal effects induced during the preincubation with CSA (data not shown). These findings contrast sharply with the pattern observed in Jurkat T-cells treated in parallel. As expected, only minimal luciferase activity was detected in untreated Jurkat cultures. Jurkat cultures treated with ionomycin and PMA demonstrated a large, 54-fold increase in activity, which was inhibited completely by pretreatment with CSA (data not shown).

**DISCUSSION**

Although CSA has been recommended for use as an adjuvant to CEV therapy in the clinical management of advanced or refractory RB, the mechanism of action of this agent in RB remains incompletely understood. Significant evidence supports the view that CSA acts in RB by reversing P-gp-mediated multidrug resistance. However, a recent study failed to support this hypothesis, finding no correlation between P-gp expression and response to chemotherapy in RB. Our results suggest that CSA’s therapeutic effects may be attributable, at least in part, to a direct cytotoxic effect on RB cells. We found that at 72 and 96 hours after treatment, the IC50 of CSA in Y79 cells lay between 20 and 30 μg/mL, a concentration comparable to the mean peak plasma levels of CSA observed in patients with RB treated with high-dose CSA therapy (21.7 ± 6.3 μg/mL; mean ± SD). In addition, we found that CSA inhibited RB cell proliferation through induction of apoptosis.

**Effect of CSA on CN/NFAT-Mediated Signaling in Y79 Cells**

We next performed transfection studies to determine whether the CN/NFAT signaling pathway is functional in Y79 cells and to determine whether CSA can inhibit this signaling. Y79 cells were transfected with an NFAT-dependent luciferase reporter construct containing three tandem NFAT-binding sites in the promoter region. In other cell types studied, activation of CN and subsequent dephosphorylation and nuclear translocation of cytoplasmic NFAT is sufficient to promote luciferase expression from this construct, which can be quantified by a luminescence assay. Y79 cells were simultaneously cotransfected with a constitutively active β-gal expression vector. Quantification of β-gal activity by a parallel luminescence assay was used to normalize transfection efficiency across replicate experiments (data not shown).

After transfection, cells were treated at selected time points with ionomycin and PMA or with CSA followed by ionomycin and PMA. Ionomycin and PMA are widely used reagents that activate CN/NFAT-mediated transcription in other cell types, and preincubation with CSA inhibits stimulation of CN/NFAT activity by these agents. Three groups of transfected cultures were treated according to the following schemes: Group 1 was stimulated with ionomycin and PMA at 48 hours after transfection. Group 2 was treated with 50 μg/mL CSA at 47 hours after transfection and stimulated with ionomycin and PMA at 48 hours after transfection (i.e., after 1 hour of preincubation with CSA). Group 3 was treated with 50 μg/mL CSA at 30 hours after transfection and stimulated with ionomycin and PMA at 48 hours after transfection (i.e., after 18 hours of preincubation with CSA). Control cultures were treated with vehicle alone. After 3 hours of stimulation with ionomycin and PMA (at 51 hours after transfection) cells were lysed, and the luciferase activity was quantified by luminescence assay.

These experiments demonstrated a high endogenous luciferase signal in untreated Y79 cultures, which was only minimally augmented by addition of ionomycin and PMA to the culture media (Fig. 6, first bar), indicating that NFAT is constitutively active in these cells. Pretreatment of Y79 cultures with CSA 1 hour before stimulation with ionomycin and PMA abrogated the stimulatory effect of these agents and also resulted in a 30% reduction in luciferase signal intensity compared to vehicle-treated controls (Fig. 6, second bar). Longer incubation with CSA, for 18 hours before treatment with ionomycin and PMA, resulted in a 94% reduction in luciferase activity (Fig. 6, third bar). No significant reduction in β-gal activity was observed in cells pretreated with CSA, indicating that the attenuation of luciferase signal could not be attributed simply to cytosstatic or cytoidal effects induced during the preincubation with CSA (data not shown). These findings contrast sharply with the pattern observed in Jurkat T-cells treated in parallel. As expected, only minimal luciferase activity was detected in untreated Jurkat cultures. Jurkat cultures treated with ionomycin and PMA demonstrated a large, 54-fold increase in activity, which was inhibited completely by pretreatment with CSA (data not shown).

**DISCUSSION**

Although CSA has been recommended for use as an adjuvant to CEV therapy in the clinical management of advanced or refractory RB, the mechanism of action of this agent in RB remains incompletely understood. Significant evidence supports the view that CSA acts in RB by reversing P-gp-mediated multidrug resistance. However, a recent study failed to support this hypothesis, finding no correlation between P-gp expression and response to chemotherapy in RB. Our results suggest that CSA’s therapeutic effects may be attributable, at least in part, to a direct cytotoxic effect on RB cells. We found that at 72 and 96 hours after treatment, the IC50 of CSA in Y79 cells lay between 20 and 30 μg/mL, a concentration comparable to the mean peak plasma levels of CSA observed in patients with RB treated with high-dose CSA therapy (21.7 ± 6.3 μg/mL; mean ± SD). In addition, we found that CSA inhibited RB cell proliferation through induction of apoptosis.
Although penetration of CSA across the blood-brain barrier (and by inference, the blood-retinal barrier [BRB]) is typically limited, a variety of evidence suggests that RB cells may be exposed to CSA levels comparable to those found in plasma. The development of RB has been associated with breakdown of the BRB. Electron microscopy studies of vascular elements in RB reveal features consistent with BRB disruption, including loss of endothelial junctions and fenestrae. Disruption of the BRB is especially pronounced in advanced RB, as demonstrated by fluorescein leakage and hyperfluorescence of tumor on fluorescein angiography. Further evidence of increased BRB permeability in RB is provided by a report of intravitreal carboplatin concentrations in patients with RB that were 13 times higher than intravitreal carboplatin concentrations observed in healthy primates treated with the same dose. Because vitreal penetration by carboplatin occurs through passive diffusion, it is likely that intraretinal carboplatin levels in these patients were considerably higher, especially adjacent to vascular structures where RB cells tend to proliferate. Taken together, these findings suggest that RB patients receiving high-dose CSA therapy may sustain intraretinal levels of CSA sufficient to exert clinically meaningful toxic effects on RB cells.

To test whether the antiproliferative and apoptotic effects of CSA in RB cells are mediated by inhibition of CN, we compared the effects of CSA and its analogue, PSC-833, in Y79 cells. As expected, we found that the antiproliferative and proapoptotic effects of PSC-833 were markedly reduced in comparison to those of CSA. The differences in apoptotic effects induced by these agents were particularly pronounced. Whereas CSA was strongly proapoptotic, PSC-833 induced minimal apoptosis and no significant necrosis, suggesting that PSC-833 may inhibit Y79 cell proliferation through induction of cell-cycle arrest. Whereas most studies report that PSC-833 is proapoptotic in neoplastic cells, other investigators have also reported that PSC-833 induces cell-cycle arrest.

Because prolonged cell-cycle arrest is commonly associated with delayed apoptosis, we speculate that examination of PSC-833-treated samples at later time points could reveal higher levels of apoptosis. Nevertheless, as both CSA and PSC-833 inhibit the activity of P-gp, but only CSA inhibits CN, we infer that the greater antiproliferative and proapoptotic effects associated with CSA treatment are due to inhibition of CN by CSA. Although the cytostasis induced by inhibition of P-gp by CSA could also contribute to the agent’s overall antiproliferative action, the marked proapoptotic effect of CN inhibition, which occurs at much lower concentrations of the agent, may obscure the effects of this less potent activity.

To determine whether the cytotoxic effects of CSA could be mediated by inhibition of CN/NFAT signaling, we investigated the expression of CN and NFAT family members in RB cells. Review of the literature revealed a single, early report of CN expression in RB, and no description of NFAT expression in RB cells. Our studies revealed the presence of both CN subunits, as well as NFATc1 and NFATc3 in Y79 cells, demonstrating that the critical components of the CN signaling pathway are conserved in RB cells. Furthermore, since NFATc3 is so highly expressed in RB cells, our findings suggest that this species could be the principle mediator of CN signaling in these cells. By transfecting RB cells with an NFAT-sensitive luciferase reporter gene, we also demonstrated high constitutive activity of this pathway in actively dividing RB cells and showed that pretreatment with CSA effectively inhibited NFAT activity. These findings reveal the functional integrity of the CN/NFAT signaling pathway in proliferating RB cultures, highlighting the sensitivity of the pathway to inhibition by CSA, and suggest that NFAT-mediated transcription may be an important factor in the growth and survival of RB cells.

Collectively, our results suggest that the observed clinical benefits of CSA in RB may be due to both MDR reversal and inhibition of CN/NFAT signaling in RB cells. We are currently performing additional in vitro and in vivo studies to refine and validate this hypothesis further. Meanwhile, clinical outcomes in patients with RB receiving adjuvant CSA therapy continue to demonstrate promise (Gallie BM, personal communication, 2004), and clinical trials of CSA therapy in RB have been proposed. A program project grant investigating the clinical utility of CSA in conjunction with CEV for children with large RB received funding from the American College of Surgeons Oncology Group. A multicenter international phase II clinical trial of CEV+CSA therapy for newly diagnosed poor-prognosis RB has also been funded by the Ontario Cancer Research Network.
As there are limited chemotherapeutic regimens available to clinicians for the treatment of intraocular RB, the need for additional anti-RB agents is great. In future studies we hope to develop alternatives to CSA that are more effective and better tolerated. For example, an agent that specifically targets NFAT could also have therapeutic efficacy in RB with fewer side effects than CSA. Such agents have already been proposed as alternatives to CSA for transplantation therapy. Future innovations will only benefit from further research into the mechanisms of action of agents with clinical efficacy in this disease.

Acknowledgments
The authors thank Su-Ling Wang for help in preparation of the figures.

References


